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RESEARCH

Determination of a safe protocol for using laser ablation with indocyanine green dye in endodontic treatment. In vitro, in vivo and human study

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Abstract This study aimed to determine a safe protocol for using Laser Ablation therapy (LA) with Indocyanine Green (ICG) to treat infected root canals.

Methods ***In vitro study*** **-** The root canal of an extracted human lower incisor was prepared to #35/.06 and was filled with 0.05% ICG dye. The λ 810 nm infrared diode laser was activated during the movement of introducing a #20 fiber into the root canals. The maximum temperature reached in the three root thirds was considered for protocols, modifying the power, pulse interval, and pulse duration. ***In vivo study*** **-** Extracted human lower incisor, lower premolar, and upper molar were selected. Their canals were prepared to #35/0.04, and the teeth were inserted into the subcutaneous tissue of Wistar rats. LA with ICG protocol selected in the in vitro study was performed on each root. The root surface temperature was measured in the three thirds. Then, the subcutaneous tissue adjacent to the roots was removed immediately or after 7 days and subjected to histological analysis in H&E stain. ***Human study*** **-** Thirteen lower incisors indicated for extraction from eleven patients were included. The canals of all teeth were prepared and received LA treatment with ICG in a similar way to the in vivo study. After tooth extraction, the apical thirds of the roots were processed for histopathological analysis.

Results The in vitro study revealed that the increase in power and pulse duration, or the reduction in pulse interval, determined the temperature rise. The protocol with the highest power that showed a temperature increase below 40 °C was the 2.5 W (power), 300 ms (interval), and 100 ms (duration). The in vivo study revealed that the greatest temperature variation was observed in the lower incisor; for this reason, it was selected for the human study. In the human study, a structure of the periodontal ligament with histological and morphological characteristics comparable to those of healthy tissues and similar to the roots of control teeth was observed in the treated teeth ($p<0.05$). No harmful effects were observed on the cementum structure, periodontal ligament, and alveolar bone in any teeth evaluated.

Conclusions It was concluded that the LA protocol used is safe and can be performed during the treatment of infected root canals.

Keywords Laser ablation · Laser absorption · Cementum, periodontal ligament · ICG, 810nm wavelength diode laser

Introduction

The root canal system contamination, associated with dental pulp necrosis, triggers a periapical immune response that leads to apical periodontitis [1]. The endodontic treatment aims to reduce the microbial load in the root canal system of teeth with pulp necrosis to create conditions for the body to repair the region [2]. The primary therapeutic strategy used for this purpose is the biomechanical preparation of the root canal, using irrigating solutions such as sodium hypochlorite, intracanal medication based on calcium hydroxide, and the three-dimensional filling [3]. After these procedures, a

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significant reduction in the microbial load of the root canal system has been proven [4]. On the other hand, different studies indicate that even the correct execution of these strategies cannot eliminate microorganisms from the root canal system [5], and, unfortunately, endodontic treatments present failure rates, which vary according to the study and evaluation criteria [6, 7].

Cases of failure after endodontic treatment can be due to several causes, such as instrumentation failures, inadequate use of irrigating solutions, difficulties in cleaning and disinfection, poor quality in root canal filling, coronal infiltration, untreated canals, and systemic influences on the individual's condition, among others [5, 7, 8]. Despite several factors related to failure, microorganisms are present in all of them. Thus, the main difficulty is that microorganisms lodge in the anatomical complexity of the root canal system, hindering the action of endodontic instruments and being little affected by irrigating solutions and intracanal medication [9, 10]. Thus, the success of endodontic treatment depends on the adequate cleaning and shaping of the root canal system, as well as the maximum elimination of microorganisms from the root canal, pulp tissue, and necrotic remains [2, 11]. Given these limitations, new adjuvant therapeutic strategies have been investigated to enhance cleaning and reduce the residual microbial count in root canal systems, thereby increasing the success rate of endodontic treatment [12, 13].

In recent decades, different technologies have emerged, one of the most relevant being lasers [13]. Laser ablation (LA) enhances microbial reduction following chemomechanical preparation of root canals [14]. This therapy carbonizes the tissue, killing microorganisms inside the root canal [12]. In this technique, indocyanine green (ICG) has been indicated to improve the effectiveness of ablation [15]. The ICG dye receives laser power, causing damage to tissues and cells that are in contact with the photosensitized agent [16, 17].

LA is a two-step treatment that involves applying and retaining a photosensitizing compound in the target tissues, which is then activated by exposure to light at an appropriate wavelength [14]. ICG is fundamental to the LA technique in the root canals, as it is different from other photosensitizers, as it has a photodynamic effect of 20% [18] since its absorption range is between 600 nm and 900 nm and emits fluorescence between 750 nm and 950 nm [19] coinciding with commercially available laser diodes for dental use [14, 20–22]. Its main action is through the photothermal effect due to its ability to absorb laser radiation energy [17]. The power and the duration of the pulses in milliseconds combine the irradiation and temperature of the laser [20]. The ICG absorbs irradiation, raising the local temperature and consequently evaporating it. After irradiation, the photosensitizer transitions from the low-energy singlet level "ground state" to a higher energy state 'triple state' [14].

A first study evaluated the use of ICG in LA with an infrared diode laser on *E. faecalis* biofilms in root canals and demonstrated its efficiency [14]. This study opens up new perspectives for LA therapy with ICG as an adjuvant treatment in infected root canals. In this way, a recent clinical study evaluated 60 patients with periapical lesions, where, after canal instrumentation, the patients received this therapy. According to the results, the treatment significantly increased bacterial reduction in the infected root canals, regardless of the instrumentation size or the irrigation solution used [12].

Despite the promising results obtained with LA with ICG, a significant problem related to using medium and high-power lasers is the possible heating and the possibility of high temperature causing damage to the tooth-supporting tissues [23]. There are studies related to heat production during dental procedures and the harmful effects [23, 24]. Raising the temperature on the external surface of the root can cause damage to this region, including inflammation and disorganization of the periodontal ligament, resorption of dentin and cementum, and damage to the adjacent bone tissue [24].

On the other hand, no clinical studies have evaluated possible histological changes in the periodontal ligament resulting from LA. Since the LA temperature increases due to the carbonization of the tissues, this fact motivated us to carry out this study.

The present study aimed to determine a safe protocol for using LA with ICG to treat infected root canals. To this end, we analyzed in vitro the increase in external temperature of the root surface in different LA protocols with ICG. Next, we analyzed in vivo the temperature variation and tissue response of an LA protocol with ICG, selected from the in vitro study, in different types of teeth. Finally, we analyzed in humans the protocol selected in the in vitro study and the chosen tooth type in the in vivo study.

Materials and methods

The in vitro, in vivo, and human studies were conducted in accordance with the ethics committee guidelines for each type of study. Animal procedures were approved by the Ethics Committee (CEUA 00388–2020) and followed the UK Animals (Scientific Procedures) Act 1986. The human study was approved by the Ethics Committee (CAAE 70009423.2.0000.5539). The flowchart summarizing the study process, from in vitro to in vivo and human experiments, is presented in Fig. 1.

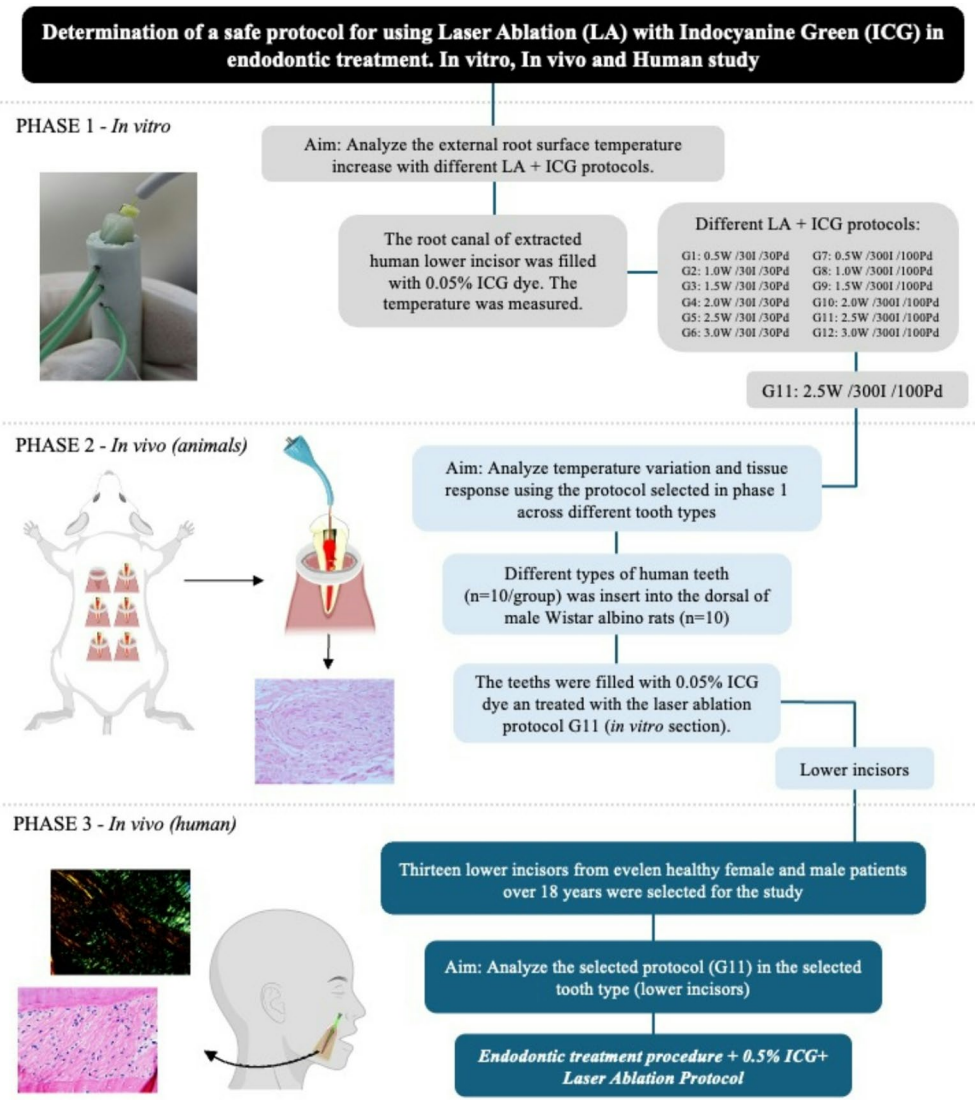

Fig. 1 Flowchart of In Vitro, In Vivo and Human studies

In vitro study

Endodontic treatment procedure

An extracted human lower incisor was selected. After coronary access, the root canals were prepared with a TDK File #35/.06 reciprocating instrument (Eurodonto - Importação e Exportação Ltda.).

The root canal was irrigated with 2.5% NaOCl using a syringe (Descarpack, São Paulo, Brazil) and an irrigation needle, Navitip (Ultradent, Utah, USA). At the end of preparation, the root canal received the following final irrigation protocol: 10 ml of sodium hypochlorite, with 30-second activation with an E1 Helse ultrasonic insert, coupled to an ultrasound device PM 200 EMS at 10% power. Second activation with 10 ml of EDTA-T for 30 s with the same parameters. The third activation, using 10 ml of NaOCl for 30 s, had the same parameters.

Sample Preparation

A round drill was used to prepare 3 cavities in the apical, middle and cervical thirds of the tooth roots. The foramen was sealed with red wax, so as not to leak the irrigating solution, and the teeth were wrapped in an additional silicone to simulate the periodontal ligament [23]. Three temperature sensors (K-type thermocouples) were coupled to the cavities prepared in the three root thirds (Fig. 2). The other ends of the sensors were connected to a digital Thermometer Data Recorder MTM-380SD device (Lutron Electronic Enterprise Co., Ltd, Taipei City, Taiwan) to read the temperature. The teeth were placed inside an oven so that their

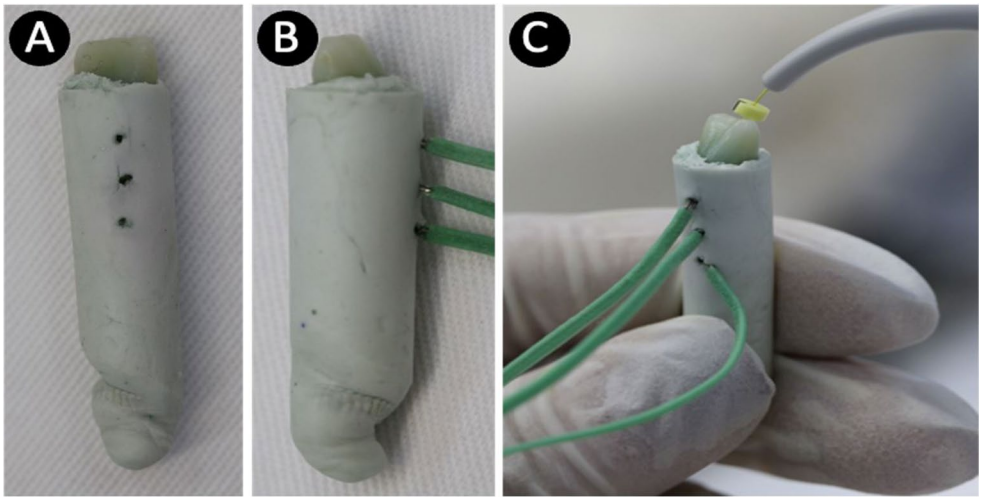

Fig. 2 The experimental model used for the in vitro study is as follows: **A** a lower incisor wrapped with additional silicone; **B** sensors attached to the 3 thirds of the root; **C** laser fiber inserted at working length.

initial temperature was approximately 37 °C to simulate body temperature.

LA and temperature measurement

The root canal was filled with 0.05% ICG dye (MP Biomedicals - Thermo Fisher Scientific, Pittsburgh, PA), and it remained for 1 min (pre-irradiation time). After this period, a #20 laser fiber was inserted into the root canal to the working length. Then, an 810 nm wavelength infrared diode laser (Picasso Plus Diode Laser (AMD Lasers, West Jordan, UT) was activated using different protocols, modifying the power, pulse interval, and pulse duration. Circular movements were performed for 30 s, together with the movement of removing the laser fiber. Then there was a 30-second pause, followed by a new 30-second activation cycle.

Different LA protocols were used with ICG by changing the power (W)/the Interval (I) in milliseconds/and the Pulse duration (Pd) in milliseconds:

- G1: 0.5 W/30I/30Pd;
- G2: 1.0 W/30I/30Pd;
- G3: 1.5 W/30I/30Pd;
- G4: 2.0 W/30I/30Pd;
- G5: 2.5 W/30I/30Pd;
- G6: 3.0 W/30I/30Pd;
- G7: 0.5 W/300I/100Pd;
- G8: 1.0 W/300I/100Pd;
- G9: 1.5 W/300I/100Pd;
- G10: 2.0 W/300I/100Pd;
- G11: 2.5 W/300I/100Pd;
- G12: 3.0 W/300I/100Pd.

After each protocol, the canal was washed with 5 mL of saline solution and aspirated with an aspirator cannula. During the LA procedure with ICG, temperature was monitored using a Thermometer Data Recorder, Lutron MTM-380SD (Lutron Electronic Enterprise Co., Ltd, Taipei City, Taiwan), which records each temperature change. The maximum temperature reached in the 3 root thirds was considered for each protocol.

In vivo study

Teeth selection and Preparation

Ten lower incisors, ten lower premolars, and ten upper molars were collected from patients requiring tooth extraction who attended the University under consent. The teeth had their crowns removed using a precision diamond saw (Isomet 1000). The roots of the lower incisors, lower premolars, and the mesial and distal roots of the upper molars were used, totaling 40 roots ($n = 10$ per group). The canals were prepared with a reciprocating instrument TDK File #35/.06 (Eurodonto - Importação e Exportação Ltda.), similar to the in vitro procedure. After preparation, the canals were washed with 5 ml of distilled water, aspirated with suction cannulas, and dried with absorbent paper cones. Then, the specimens were placed in plastic tubes and taken to an autoclave sterilization cycle [25].

Animal model

Ten male Wistar albino rats (200–250 g/2–3 months old) were used [26]. The rats were anesthetized by intramuscular administration of ketamine hydrochloride 10% (Cetamin, Syntec), 80 mg/kg, and xylazine 2% (Xylazin, Syntec), 10 mg/Kg [27]. The dorsal region was then shaved and a 1.0 cm incision was made in the lateral direction for each implant. The skin was dissected to create a pocket in the connective tissue. Six pockets were created in each animal, using the midline of the dorsal region as a reference for the creation of

three pockets on each side. Five pockets received the roots and one pocket was used for the control group. The control group was included to simulate surgical trauma and serve as a parameter for comparison with the other groups. A tooth was inserted into the pocket; the electrodes were inserted into the skin and positioned in contact with three-thirds of the root. The canal was washed with saline solution and dried with sterilized absorbent paper tips (Fig. 3).

LA and temperature measurement

All teeth were treated with the LA protocol G11, described in the in vitro section. During the LA procedure with 0.05% ICG dye (MP Biomedicals—Thermo Fisher Scientific, Pittsburgh, PA), temperature was monitored in all roots in the cervical, middle, and apical thirds using the Thermometer Data Recorder, Lutron MTM-380SD (Lutron Electronic Enterprise Co., Ltd, Taipei City, Taiwan).

Histological analyses

After completing the laser protocol, half of the animals were euthanized, and the subcutaneous tissue from the pocket was collected for histological analysis. The other half had their teeth removed from the pocket, and the tissue was sutured. These animals received 150 mg/kg of Dipyrone intraperitoneally for pain relief, and after 7 days, they were euthanized for tissue collection. In both periods, the animals were euthanized with Sodium thiopental anesthetic (Thiopentax, Cristalia Produtos Quimicos Farmaceuticos Ltda.), and the tissues were removed and fixed in a 10% formaldehyde neutral pH buffered solution. Then, the specimens were processed for histological analysis at H&E [28].

Histopathological analysis was performed by a single calibrated operator (C.C.S.) in a blinded manner under light microscopy 50x and 400x, Leica DM 4000 B (Leica Microsystems, Nussloch, Bade-Württemberg, Germany) [29]. A descriptive analysis of the tissues was carried out to evaluate tissue damage depending on the dental group that received the LA protocol and the immediate or late time after the protocol.

Human study

Study design and population

All procedures were conducted at the Unicesumar Dental Clinic under standardized clinical protocols. The sample consisted of thirteen lower incisors obtained from eleven healthy female and male patients over 18 years old who needed tooth extraction. Patient selection was carried out through anamnesis and clinical/radiographic examinations.

The inclusion criterion was lower incisors with an indication for extraction. The exclusion criteria were: other dental groups; teeth with periodontal disease beyond the middle third of the root; and teeth that did not require tooth extraction. Ten teeth were included in the treatment group, and three additional teeth served as a control group.

Endodontic treatment procedure

Patients were anesthetized with 3% Mepivacaine with 1:100,000 epinephrine (Mepiadre, DFL, RJ, Brazil), and the teeth were isolated with a rubber dam. The operative field was then disinfected with a povidone-iodine PVPI solution. After coronary accesses, the root canals were instrumented with a TDK File #35/.06 reciprocating instrument (Eurodonto - Importação e Exportação Ltda.). All root canals received irrigation with 2.5% NaOCl, a syringe (Descarpack, São Paulo, Brazil), and an irrigation needle Navitip

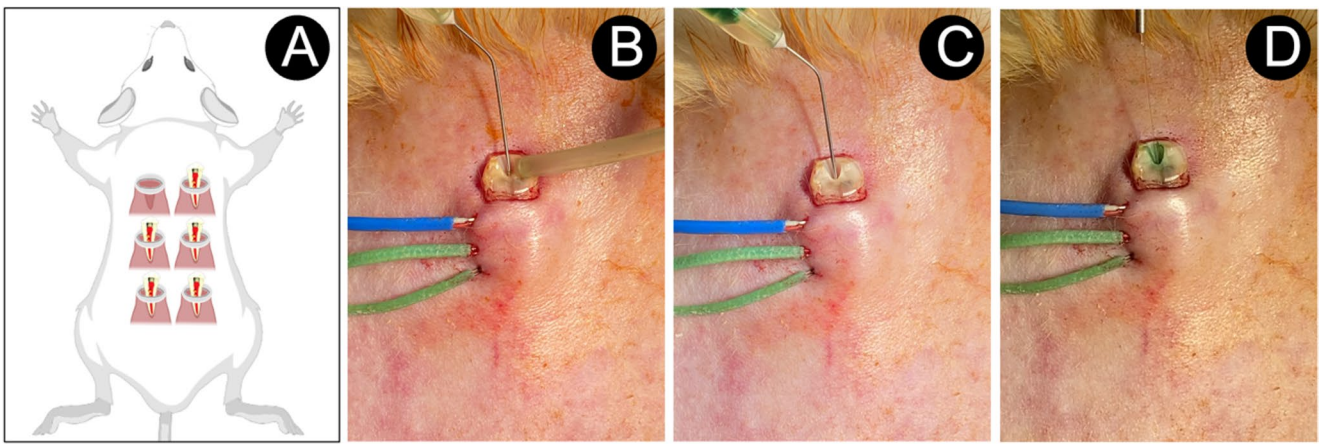

Fig. 3 Experimental model used for the in vivo study: **A** Schematic of the dental implant in the subcutaneous tissue of the rat; **B** Temperature sensors positioned in the three-thirds of the root and irrigation and aspiration procedure in the root canal; **C** Root canal filling with ICG; **D** Laser fiber positioned in the root canal for LA procedure

(Ultradent, Utah, USA). At the end of preparation, each root canal received the final irrigation protocol, similar to that used in the in vivo study.

LA protocol

After chemo-mechanical preparation, the root canals received LA therapy: (1) the root canals were filled with 0.05% ICG dye that remained in the root canal for 1 min; (2) the λ 810 nm infrared diode laser was activated during the movement of introducing a #20 fiber into the root canals; (3) circular movements were performed for 30 s together with the movement of removing the laser fiber; (4) 30 s pause; (5) new 30 s activation cycle; (6) the root canals were washed with 5 ml of saline solution, aspirated with an aspirator cannula and dried with paper cones.

Tooth extraction and histological processing

Immediately after applying LA with ICG, an incision was made in the gingival tissue around the tooth to be extracted, which was gently moved away to expose the tooth neck. Then, with an atraumatic extraction kit, the dental element was dislocated and removed using forceps and an extractor. Finally, a suture was placed to bring the edges of the gingival tissue around the socket closer together.

The apical part of the root was obtained from the dental element and the periodontal ligament. The specimens were placed in bottles containing 10% formalin solution and buffered with neutral pH during the first 20 h. They were washed and demineralized in 10% EDTA [29] for approximately 3 months. After this period, they were washed, dehydrated in alcohol, clarified in xylene, and embedded in paraffin. The paraffin blocks containing the specimens were cut with semi-serial cuts, 3 µm thick, carried out using a microtome Leica model RM 2045. For each specimen, five slides with three tissue sections were prepared and stained with H&E and Picrosirius red (PSR) [30].

Analysis of histological results

Analyses were performed at 400x magnification by a single, calibrated, and blinded operator (LTAC). The results were presented through descriptive and qualitative analysis to characterize the morphological structures of the periodontal ligament and identify possible pathological alterations. The analysis considered: the inflammatory infiltrate in terms of its intensity and extent; the organization of the periodontal ligament; the periodontal ligament space; the maturation of collagen fibers; inflammatory and/or replacement resorption; and dental ankylosis. The two control teeth were used as parameters to analyze possible morphological changes.

The inflammatory infiltrate, when present, was classified according to the number of inflammatory cells present, as follows: none or few cells (Score 0); less than 25 cells (Score 1, Mild); between 25 and 125 cells (Score 2, moderate); more than 125 cells (Score 3, severe) [31]. The arrangement of the collagen fibers was assessed to analyze the organization of the periodontal ligament. An organized periodontal ligament is characterized by collagen fibers that connect directly to the alveolar bone. The areas were measured and transformed into percentages of the organization, where: total organization (Score 0); organization greater than 75% and less than 100% (Score 1); organization between 50% and 75% (Score 2); organization less than 50% (Score 3).

The periodontal ligament space was measured to determine the possible presence of edema that could displace the tooth and allow the periodontal ligament space to increase [32]. Eight measurements were taken on each histological section, and the average of the measurements was calculated for each specimen. For this analysis, 100x magnification was used, and the measurement was carried out using the LAS Leica program (Leica Microsystems, Nussloch, Baden-Württemberg, Germany).

To resorption, gaps on the root surface with the presence of inflammatory cells in the area were defined as inflammatory resorption; gaps obliterated by bone were defined as replacement resorption and areas with an absence of periodontal space (direct contact between the root surface and adjacent bone tissue) were defined as ankylosis [33]. The areas were analyzed and considered according to their extension: no presence of pathological changes (Score 0); change in area of less than 25% (Score 1); change in area between 25% and 50% (Score 2); change in area greater than 50% (Score 3).

Statistical analysis

SigmaPlot 12.0™ software (SYSTAT Software) was used to analyze the data statistically. First, the Shapiro-Wilk normality test was performed. Then, the Mann-Whitney U test was used for non-parametric data, and the Student's t-test was used for parametric data. For all tests, a significance level of 5% was considered.

Results

In vitro study

Temperature analysis

The results of temperature measurement on the root surface in each root third can be seen in Table 1.

Table 1 LA protocols and external temperature values obtained in each root third

Groups	Laser calibration			Maximum temperature		
	Power (W)	Pulse Interval (I)	Pulse duration (Pd)	Cervical	Middle	Apical
G1	0.5	30	30	34.4	36.3	39.6
G2	1.0	30	30	34.8	36.8	44.3
G3	1.5	30	30	35.5	36.9	44.4
G4	2.0	30	30	35.7	38.2	45.5
G5	2.5	30	30	35.8	38.2	45.9
G6	3.0	30	30	36.1	38.7	46.7
G7	0.5	300	100	34.5	34.8	36.9
G8	1.0	300	100	35.5	35.7	37.6
G9	1.5	300	100	35.8	36.0	38.1
G10	2.0	300	100	35.9	36.1	38.3
G11	2.5	300	100	36.0	36.5	39.7
G12	3.0	300	100	36.3	37.1	45.0

The in vitro study revealed that the increase in power, as well as the increase in pulse duration or reduction in pulse interval, determined the increase in temperature. The protocol with the highest power that showed a temperature increase below 40 degrees Celsius was the 2.5 W/300 I/100 Pd (G11). Therefore, this protocol was used for the other experiments. Another important information from this study was that the apical third presented the greatest increase in temperature. Thus, in in vivo and human studies, the living tissues analyzed were those located in this region of the root.

In vivo study

Temperature analysis

The temperature variation in each root third for all roots can be observed in Fig. 4. The lower incisor tooth showed greater temperature variation between the root thirds (Fig. 4A). The premolar suffered the least variation between the thirds (Fig.4B), followed by the mesial (Fig. 4C) and distal root of the first molar (Fig. 4D).

When the laser was activated at 0 s, the temperature began to rise on average after 7 s. After that, the temperature rose similarly in all thirds, maintaining a variation pattern of 0.2°C in 3–4 s. After the first 30 s of activation, the temperature increases until the first 10 s of pause. After this period, the temperature decreases until the laser is activated again. The maximum temperature variation was 1.7 °C for the first laser activation. The 30-second pause reduced the temperature in all root thirds, and all roots were evaluated. At the end of the 90-second protocol, the minimum temperature variation achieved was 1.3 °C, and the maximum was 2.7 °C.

The distances between the temperature probe and the root canal in different teeth and root thirds are shown in Table 2. After temperature analysis, the teeth were removed, and using a digital caliper (Mitutoyo Sul Americana Ltda, Jundiaí, SP, Brazil), the measurement of each probe was taken up to the inside of the root canal.

In all roots, the shortest distance was observed in the apical third, and with a progressive increase until the cervical third. The smallest thickness observed was in the apical third of the lower incisor, while the greatest thickness was observed in the cervical third of the lower premolar.

Histological analysis in connective tissue

Discreet tissue changes were observed in all groups in the immediate period (Fig. 5a1-e1).

The control group (Fig. 5a1) presented slightly disorganized tissue and mild vascular changes, probably due to surgical trauma. The connective tissue adjacent to the lower incisor, as well as the mesial root of the first molar, showed a slightly more disorganized connective tissue with vascular changes in the region of direct contact with the tooth (Fig. 5b1, d1). The connective tissue adjacent to the premolar root and the distal root of the first molar showed less disorganization, with little vascular change (Fig. 5c1, e1). At 7 days, no changes in connective tissue were observed in either group (Fig. 5a2-e2). No tissue necrosis and changes in the deeper layer of connective tissue were observed in any of the periods and groups.

Human study

Histological analysis

All the treated teeth showed a periodontal ligament structure with histological and morphological aspects comparable to healthy tissues and similar to the roots of the control teeth (Fig. 5). No significant concentration of inflammatory cells was observed in any of the teeth evaluated (Fig. 5a1-a4, b1-b4; Table 3). There was no migration of

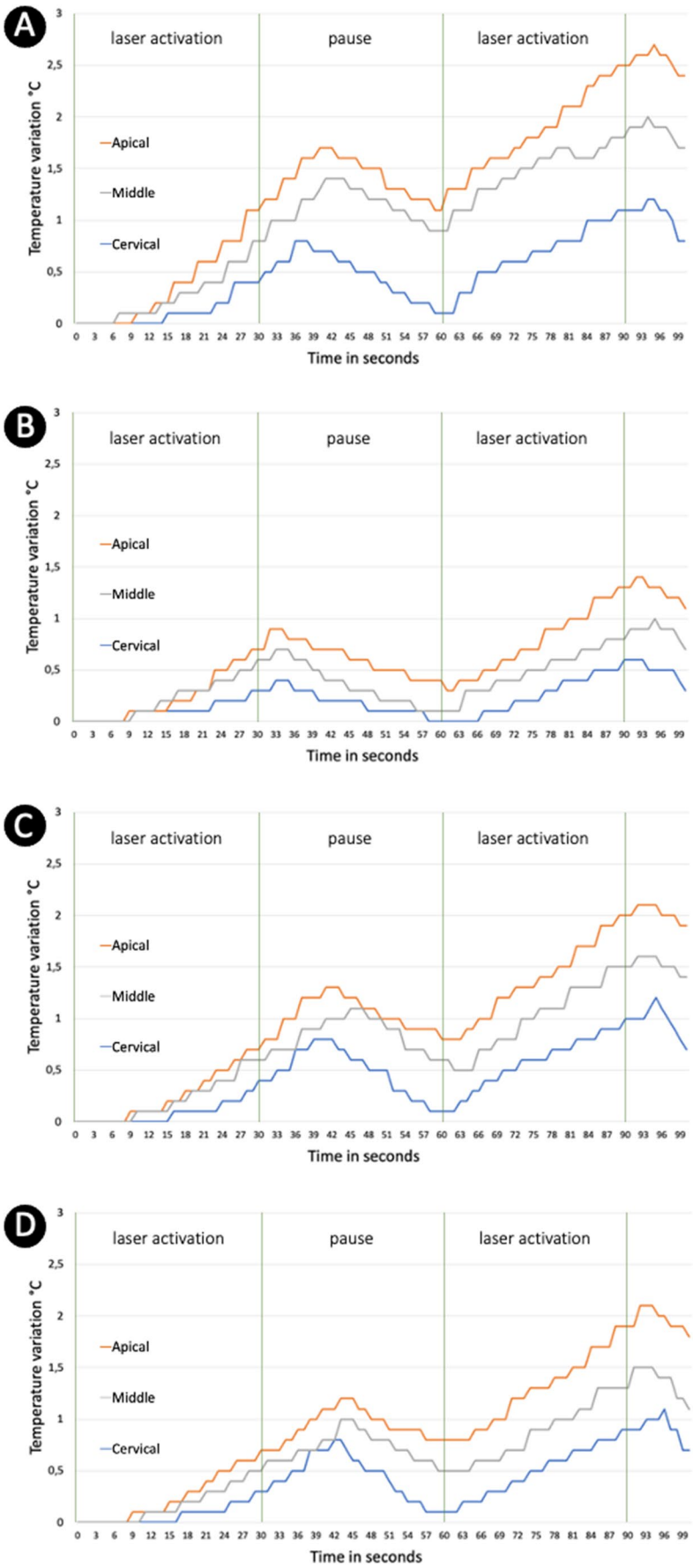

Fig. 4 Graphs with temperature variation in each root third as a function of time: **A** lower incisor; **B** lower premolar; **C** mesial root of the upper first molar, and **D** distal root of the upper first molar, respectively

Table 2 Mean and standard deviation (SD) of distances between the temperature probe and the root Canal in different groups and root thirds

Teeth/Root		Third of the root	Measure (mm ± SD)
Lower incisor		**Cervical**	1.71 ± 0.22
		Middle	1.28 ± 0.18
		Apical	0.95 ± 0.16
Lower premolar		**Cervical**	2.62 ± 0.29
		Middle	1.94 ± 0.21
		Apical	1.57 ± 0.17
Upper first molar	**Mesial root**	**Cervical**	1.78 ± 0.19
		Middle	1.33 ± 0.25
		Apical	1.25 ± 0.18
	Distal root	**Cervical**	1.82 ± 0.23
		Middle	1.49 ± 0.19
		Apical	1.22 ± 0.15

polymorphonuclear cells (neutrophils). There was a small, non-significant presence of mononuclear cells (lymphocytes and plasma cells) in both groups, which were dispersed in the periodontal ligament.

In all the tissue sections of the specimens, a 100% organized periodontal ligament was observed. The collagen fibers were integral and directly connected to the cementum of the alveolar bone (Fig. 6a1-a8, b1-b8; Table 3). As well as the absence of edema and plasma proteins. There was no statistical difference in the periodontal ligament space between groups.

In the cementum and alveolar bone region, no deleterious or harmful effects on the structures were observed in any of the teeth evaluated (Fig. 6a1-a4, b1-b4; Table 3). There is no resorption and obliterated lacunae by alveolar bone, which could indicate a process of resorption by replacement. In addition, no areas of dentoalveolar ankylosis were found.

Mann-Whitney U test was used for non-parametric data, and the Student's t-test.

Discussion

This study evaluated, in vitro, the increase in the external temperature of the root surface in different LA protocols with ICG; in vivo, the temperature variation and tissue response of the LA protocol with ICG selected from the in vitro study, in different types of teeth; in humans the periodontal ligament, cementum and alveolar bone after carrying out the protocol selected in the in vitro study and the type of tooth selected in the in vivo study. Given the results observed, a safe LA protocol with ICG can be determined as an adjuvant therapy to endodontic treatment.

Many preliminary studies, such as the previous in vitro study, are proposed to obtain information that helps to choose, among many groups, the best combination of treatments or the one that can induce fewer side effects [34]. The major problem with using a laser in intracanal therapy is the possibility of heating the external surface of the root, especially when using higher power [23, 35]. The objective of the previous in vitro study was to exclude combinations of power, pulse interval, and pulse duration that would determine large temperature increases on the external surface of the dental element.

The literature shows that the higher the laser power, the greater the proportional disinfection [36], so there is a tendency to use laser equipment with medium to high power [37]. Heating the external surface of the root above 10 °C for more than one minute should be avoided, as this is the limit level for the survival of the periodontal ligament and bone tissue [38]. On the other hand, temperature increases above 40 degrees Celsius can already cause changes to the dental supporting tissues [39], contraindicating the procedure. Based on the findings of the previous in vitro experiment, the protocol with the highest power that showed an increase in temperature below 40 degrees Celsius was with a power of 2.5 W, an interval of 300 milliseconds, and a duration of 100 milliseconds.

The in vivo study analyzed different types of teeth and, therefore, different types of roots. It was possible to observe that the thickness of the dentin influenced the delay between the light pulse and the increase in temperature. This relationship can be taken into account according to the different types of teeth and their thickness [40]. On the other hand, other factors can also delay the heating of the external surface of the root, such as calcification of the dentinal tubules and the presence of a smear layer on the internal wall of the root canal. Given the findings from the in vivo study, we selected the type of tooth that provided the greatest increase in external temperature for the human study.

Thus, data from the in vivo study made it possible to select a type of root that allowed the greatest increase in external temperature. In this case, the lower incisors are the elements with the smallest thickness of root dentin [41] and were selected for study in humans.

Additionally, an important piece of information was the identification of a protocol that made it possible to contain the increase in temperature to levels that were not harmful to living tissues [39]. Carrying out this pre-clinical study also aimed to provide security for the study in humans [42].

The changes in living tissues observed from the in vivo study were discrete and are directly related to the surgical trauma caused by the implantation of the roots. Only in the lower incisor group and in the mesial root group of the upper molar was there a slight change that was slightly higher than in the control group. This observation may be directly related to the slight increase in temperature in the area of the traumatized tissue. This condition cannot be extrapolated to

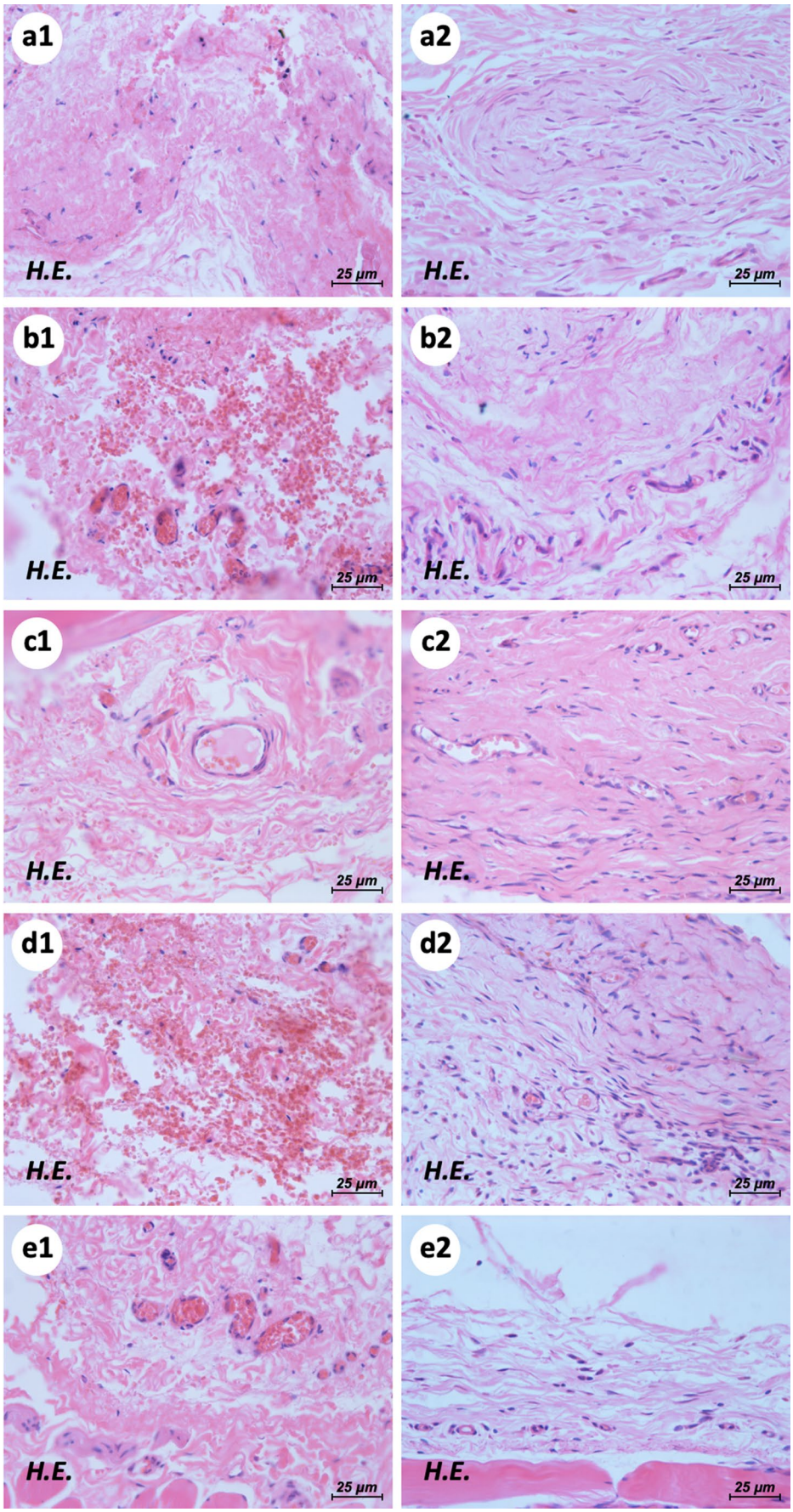

Fig. 5 (**a1**,**a2**) control; (**b1**,**b2**) lower incisor; (**c1**,**c2**) lower premolar; (**d1**,**d2**) mesial root of the upper first molar and (**e1**,**e2**) distal root of the upper first molar. Immediate (**a1-e1**) and 7-day (**a2-e2**) periods (Original magnification of x400, H&E stain)

Table 3 Scores, median, and p-values for histological analysis criteria

Analysis Parameters		Groups		*p* Value
		Control	LEAP	
Inflammatory Infiltrate	**0 - Absent**	3/3	10/10	Mann-Whitney U test $p=1.0$
	1 - Mild	0/2	0/10	
	2 - Moderate	0/2	0/10	
	3 - Severe	0/2	0/10	
	Median*	0^A	0^A	
Periodontal Ligament Organization	**0–100%**	3/3	10/10	Mann-Whitney U test $p=1.0$
	1 - >75% and <100%	0/2	0/10	
	2 - >50% and <25%	0/2	0/10	
	3 - <50%	0/2	0/10	
	Median*	0^A	0^A	
Periodontal ligament space (μm)		175.25 ±7.40^A	173.55 ±14.33^A	Student's t-test $p=0.859$
Inflammatory resorption	**0 - Absent**	3/3	10/10	Mann-Whitney U test $p=1.0$
	1 - <25%	0/2	0/10	
	2 - >25% and <50%	0/2	0/10	
	3 - >50%	0/2	0/10	
	Median*	0^A	0^A	
Replacement resorption	**0 - Absent**	3/3	10/10	Mann-Whitney U test $p=1.0$
	1 - <25%	0/2	0/10	
	2 - >25% and <50%	0/2	0/10	
	3 - >50%	0/2	0/10	
	Median*	0^A	0^A	
Ankylosis	**0 - Absent**	3/3	10/10	Mann-Whitney U test $p=1.0$
	1 - <25%	0/2	0/10	
	2 - >25% and <50%	0/2	0/10	
	3 - >50%	0/2	0/10	
	Median*	0^A	0^A	

* Different uppercase letters represent a statistically significant difference between the groups ($p<0$ 0.05)

what occurs clinically, as the tissues of the periodontal ligament, cementum, and alveolar bone are healthy at the time of application of LA with ICG. On the other hand, within 7 days, the connective tissue had already repaired itself, and no changes were observed in either group. Therefore, the results of this in vivo study were not significant and allowed the study to be safely performed in humans.

The human study evaluated the changes in the tissues (periodontal ligament and cementum) after laser ablation protocol with indocyanine green. It was observed that the structure of the periodontal ligament with histological and morphological characteristics was comparable with healthy tissues and similar to the roots of control teeth. In the absence of polymorphonucleated cells, it was clear that there was no acute inflammatory process. The small presence of mononuclear cells (lymphocytes and plasma cells) in both groups is explained by the fact that, even under normal clinical conditions, the periodontal ligament has a small number of inflammatory cells, mainly mononuclear cells [43]. In addition, this number can vary depending on several factors, such as the masticatory force load, the presence of periodontal disease and the patient's age [32, 43]. Both groups showed 100% organization of the periodontal ligament. This finding corroborates the findings of inflammatory cells, as it is only in the presence of inflammation that the degeneration of collagen fibers occurs and, consequently, the disorganization of these fibers [44]. Furthermore, very close to normal periodontal ligament space was observed, where the average human periodontal ligament can vary, but is generally between 150 μm and 380 μm [43]. Furthermore, no harmful effects on the structure of the cementum, periodontal ligament, or alveolar bone were observed in any of the teeth evaluated.

LA therapy with ICG has been studied to increase the success of root canal treatment by further reducing the microbial load of root canals after conventional instrumentation. On the other hand, heating of the root surface is possible when using higher laser power [45]. The results obtained revealed a periodontal ligament structure with normal histology and morphology. No inflammation was observed in any of the teeth evaluated. Collagen fibers were observed connecting the cement tissue to the alveolar bone and the absence of areas of cement and bone resorption. There were no changes in the structure of the cementum, periodontal ligament, and alveolar bone in any of the teeth evaluated.

Some studies related to heat production during dental procedures have reported harmful effects on the pulp [22, 23]. However, the possibility of increasing the laser power and, at the same time, shortening the pulse allows an instant heat flame of very short duration to be produced. Additionally, the possibility of increasing the power and, at the same time, increasing the interval between pulses would allow for a cooling period after heat production [46]. Therefore, LA therapy with ICG does not use constant laser activation but rather the production of heat pulses, which may have determined the results obtained in the study. Therefore, given the results obtained, the use of LA with ICG did not produce deleterious or harmful effects on dental supporting tissues, making it safe to use as an adjuvant therapy during the treatment of infected root canals.

A limitation of the present study is the small sample size in the human control group ($n=3$), which may limit the statistical power of the comparisons. However, all histological findings were consistent across the treatment and control groups and revealed no signs of inflammation, resorption, or structural alterations in the periodontal ligament, cementum, or alveolar bone. These results align with the in vivo observations, where only mild and transient tissue changes

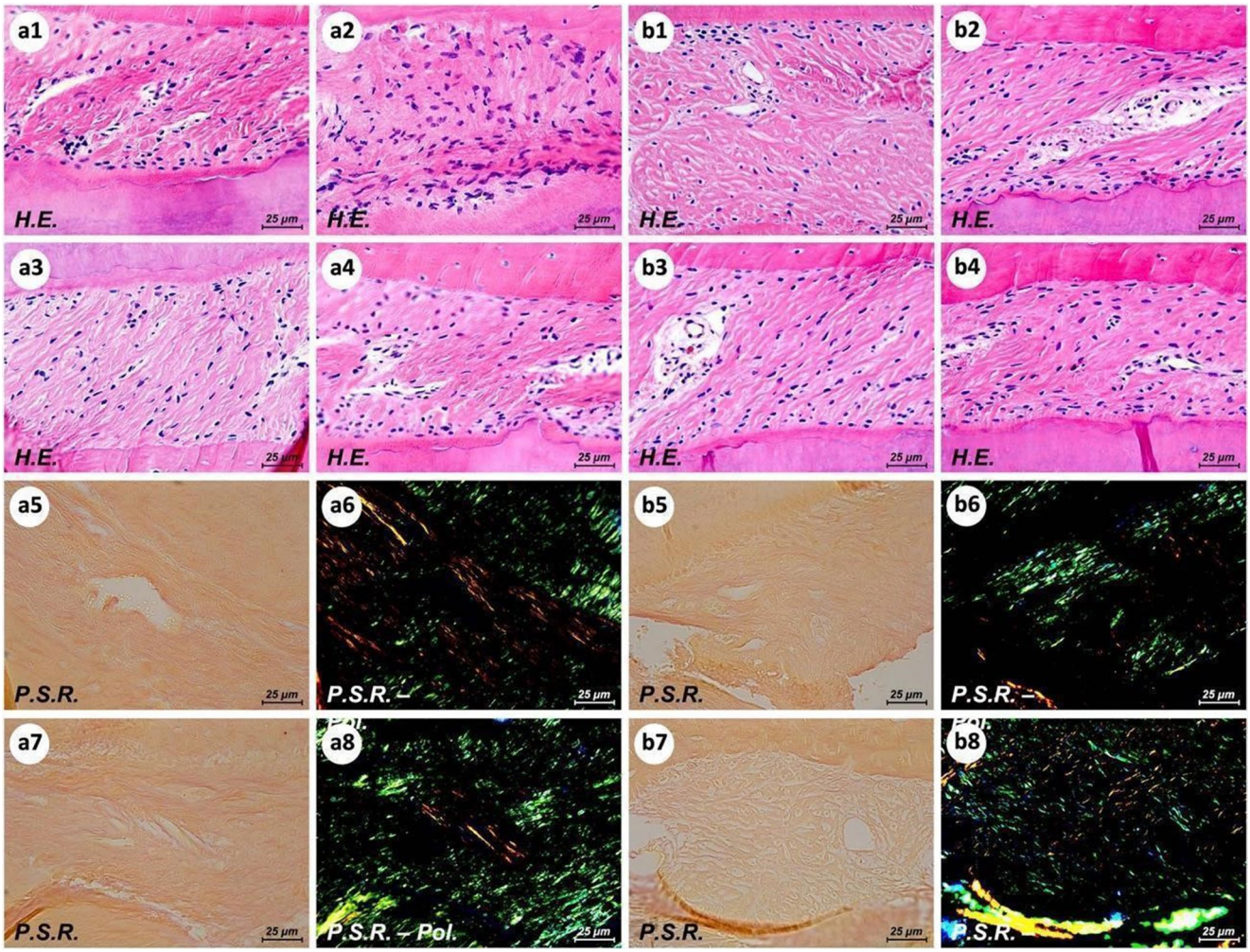

Fig. 6 Control group (**a1**-**a8**) and Treatment group (**b1**-**b8**), in Hematoxylin and Eosin stain (**a1**-**a4**, **b1**-**b4**), Picrosirius red stain (**a5**,**a7**,**b5**,**b7**), and Picrosirius red stain polarized (**a6**,**a8**,**b6**,**b8**). (Original magnification of x400, H&E, and PSR stain)

were seen, mostly attributed to surgical trauma. The in vitro analysis also reinforces the findings by confirming that the selected laser ablation protocol did not elevate external root surface temperatures above biologically critical thresholds.

Conclusions

It was concluded that LA therapy with ICG using an 810 nm diode laser at a power of 2.5 W, an interval of 300 milliseconds, and a duration of 100 milliseconds, with repetition of the cycle after a 30-second pause, is safe and can be performed during the treatment of infected root canals.

Abbreviations

LA	Laser Ablation
ICG	Indocyanine Green
H&E	Hematoxylin and Eosin
PSR	Picrosirius Red
CEUA	Comissão de Ética no Uso de Animais (Ethics Committee on Animal Use)
CAAE	Certificado de Apresentação para Apreciação Ética (Certificate of Presentation for Ethical Consideration)
NaOCl	Sodium Hypochlorite
EDTA	Ethylenediaminetetraacetic Acid
W	Watt
I	Pulse Interval (ms)
Pd	Pulse Duration (ms)
PVPI	Povidone–Iodine

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Declarations

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