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ORIGINAL ARTICLE

Laser and LED phototherapy on midpalatal suture after rapid maxilla expansion: Raman and histological analysis

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Abstract The aim of this study was to analyze the effect of laser or LED phototherapy on the acceleration of bone formation at the midpalatal suture after rapid maxilla expansion. Forty-five rats were divided into groups at 7 days (control, expansion, expansion and laser irradiation, and expansion and LED irradiation) and into 14 days (expansion, expansion and laser in the 1st week, expansion and LED in the 1st week, expansion and laser in the 1st and 2nd weeks, expansion and LED in the 1st and 2nd weeks). Laser/LED irradiation occurred every 48 h. Expansion was accomplished with a spatula and maintained with a triple helicoid of 0.020-in stainless steel orthodontic wire. A diode laser (λ780 nm, 70 mW, spot of 0.04 cm^2, $t = 257$ s, SAEF of 18 J/cm^2) or a LED (λ850 ± 10 nm, 150 ± 10 mW, spot of 0.5 cm^2, $t = 120$ s, SAEF of 18 J/cm^2) was applied in one point in the midpalatal suture immediately behind the upper incisors. Raman spectroscopy and histological analyses of the suture region were carried and data was submitted to statistical analyses ($p \leq 0.05$). Raman spectrum analysis demonstrated that irradiation increases hydroxyapatite in the midpalatal suture after expansion. In the histological analysis of various inflammation, there was a higher production of collagen and osteoblastic activity and less osteoclastic activity. The results showed that LED irradiation associated to rapid maxillary expansion improves bone repair and could be an alternative to the use of laser in accelerating bone formation in the midpalatal suture.

Keywords Phototherapy · Lasers · LED · Orthodontics · Osteogenesis · Palatal expansion technique

Introduction

Rapid maxillary expansion is considered one of the most important methods in the correction of maxillary atresia, which frequently causes posterior crossbite [1, 2]. The expansion procedure used to correct this arch deficiency is based on the orthopedic separation of the segments of the maxilla by applying forces with enough magnitude to rupture the bioelastic structures of the midpalatal suture [2]. Despite both dental and skeletal effects caused by this therapy being demonstrated previously, its efficiency is still a matter of discussion as there

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are conflicts in relation to how long and what type of retention is needed after orthodontic appliances are removed [3].

Relapse is a tendency after tissue expansion and insufficient bone regeneration in the midpalatal suture after the procedure has been appointed as one of its main causes. Therefore, a long period of retention has been indicated to prevent relapse during tissue reorganization [1]. Accelerated bone formation in the region following rapid maxillary expansion would be beneficial to avoid relapse as well as reduce the retention time [1].

Low-intensity light therapy, commonly referred to as "phototherapy," using far-red to near-infrared (NIR) light is capable of modulating numerous cellular functions [4]. The quickening of bone formation is one of the various biostimulatory effects of low-level lasers [1]. Laser efficiency in bone healing has been shown elsewhere in the literature [5–7] including speeding bone neoformation in cases of midpalatal expansion [1, 8, 9]. Laser irradiation could potentially stimulate the recruitment of osteoblasts and/or their maturation throughout the bone edges of the midpalatal suture in expansion [1]. The osteoblasts would be stimulated and increase the deposition of calcium hydroxyapatite with consequent quicker bone maturation as well as increased resistance [10]. Therefore, the laser action could lead to a shorter period of retention and to a more stable occlusion. However, most of the studies regarding the use of laser therapy combined with suture expansion used different protocols (total dosage, irradiation time, mode and frequency of irradiations), which are factors that influence the outcome of the treatment and prevent precise comparisons between studies. The use of LED light has also been reported to cause positive biostimulatory effects somewhat similar to those observed when laser light is used [10–12].

Unlike laser, LEDs emit light using spontaneous emission of radiation while lasers produce stimulated emission of radiation [13]. Another significant difference between lasers and LEDs is the way energy is delivered. LEDs provide a much gentler delivery of the same healing wavelengths of light as do the laser but at a substantially greater energy output [14]. LED is a monochromatic light source that emits into a relatively small spectral band considered a narrowband [15] but with a wider emission spectrum of the laser [16]. Thus, LED has a different spectral distribution, perhaps interacting with the largest number of photoreceptors [16, 17]. LEDs have wider angles or beams of light and greater light-scattering effects, which provide an even distribution of light energy over broader areas of treatment resulting in shorter treatment time [14]. Furthermore, phototherapy in the infrared is considered risk-free and has been FDA approved for use in humans [4]. For the patient, treatment with LED is painless, fast, and without discomfort [15].

Raman spectroscopy can provide detailed information of the chemical composition of a tissue throughout the Raman effect that involves an energy exchange between light and matter [18]. Raman scattering occurs when molecules within a specimen are excited by incident laser light and its vibrational motions within the molecules lead to small fraction of the light losing energy and being scattered at longer wavelengths [10]. From an energetic point of view, Raman scattering can be observed as a transition of the molecule from the fundamental state, with a lower level of energy, to an excited vibrational state, through the absorption of the incident photon and posterior emission of one [18]. The wavelength difference between scattered and incident light corresponds to molecular-specific vibrations called the Raman shift and leads to spectral bands that provide direct information on the biochemical composition of the sample. Raman peaks are spectrally narrow and, in many cases, can be associated to the vibration of a particular chemical bond (or a single functional group) in the molecule [10]. The Raman spectrum of bone shows prominent vibrational bands related to tissue composition [10] and has been used recently in several studies related to bone formation using different models [10, 19].

There are few studies in the literature considering the effect of laser and LED on midpalatal suture after rapid maxillary expansion and if it improves bone repair after this procedure. Therefore, the present investigation aimed to evaluate, through near-infrared Raman spectroscopy and histological analysis, the effects of laser or LED irradiation on bone repair following rapid maxillary expansion.

Materials and methods

This work has been developed according to legal and ethical specifications for animal experiments and approved by the Animal Experimentation Ethics Committee of the School of Dentistry of the Federal University of Bahia, Brazil (Protocol no. 03/10). Forty-five male Wistar rats (6 weeks old, mean weight 170 ± 20 g) were used and maintained in the Laboratory of Animal Experimentation of the School of Dentistry of the Federal University of Bahia. The animals were kept in cages containing five animals each, in a room temperature of 22 to 26 °C with day/night light cycle. Before the expansion procedures, the animals were anesthetized (0.12 ml/100 g of ketamine, Vetaset®, São Paulo, SP, Brazil, and 0.6 ml/100 g of xylazine, Coopazine®, Coopers, São Paulo, SP, Brazil).

The expansion device consisted of a triple helicoid spring made of 0.020-in stainless steel orthodontic wire (Morelli®, Sorocaba, SP, Brazil). The triple helicoid spring occupied a 1.5-mm space between the incisors when installed and had lateral hooks that served as support for resin bonding of the device to the teeth. All devices were manufactured in the same size and the triple helicoid was measured with a digital caliper. The rat's superior incisors were separated with a resin spatula and the expansion spring was installed in the midline (Fig. 1).

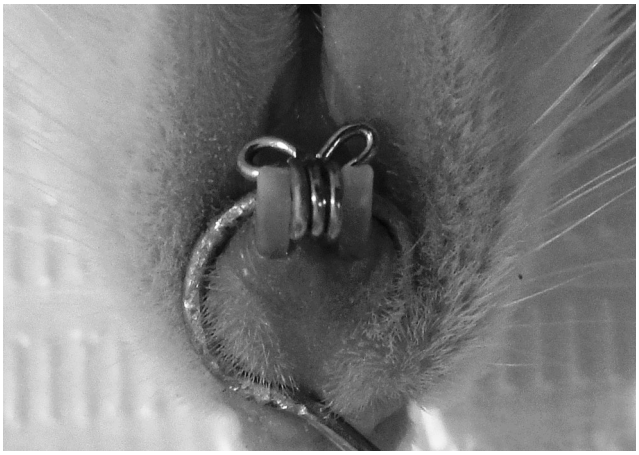

Fig. 1 The superior incisors were separated with a resin spatula and the expansion spring installed in the midline

Enamel conditioning of the incisors with 37% phosphoric acid gel (Alpha Acid®, DFL, Rio de Janeiro, RJ, Brazil) for 60 s was performed, followed by rinsing and drying of the teeth's surface for 20 s. The bonding was accomplished with an adhesive system (Magic Bond®, Vigodente, Rio de Janeiro, RJ, Brazil) and compound resin (Fill Magic®, Vigodente, Rio de Janeiro, RJ, Brazil) and simultaneously light cured for 40 s (ULTRALED XP®, Dabi Atlante, Ribeirão Preto, SP, Brazil).

The rats were divided into nine groups: 7-day groups (1—control (no treatment); 2—expansion; 3—expansion and laser irradiation; 4—expansion and LED irradiation) and groups with 14 days of experimental time (5—expansion; 6—expansion and laser in the 1st week; 7—expansion and LED in the 1st week; 8—expansion and laser in the 1st and 2nd weeks; and 9—expansion and LED in the 1st and 2nd weeks) (Table 1). In the 7-day groups, there was laser or LED application in the 1st, 3rd, and 5th experimental days (48 h intervals). In the 14-day groups, there was laser or LED irradiation in 1st, 3rd, 5th, 8th, 10th, and 12th experimental days (48 h intervals).

For phototherapy, either a diode laser (Twin Flex®, MMOptics, São Carlos, SP, Brazil, λ780 nm, 70 mW, spot of 0.04 cm^2, area of irradiation in the tissues of 1 mm^2, total irradiation dose per session of 18 J/cm^2, and 257 s of irradiation) or a LED device (FisioLED®, MMOptics, São Carlos, SP, Brazil, λ850 ± 10 nm, 150 mW, spot of 0.5 cm^2, area of irradiation in the tissues of 1 mm^2, total irradiation, dose per session of 18 J/cm^2, and 120 s of irradiation) was applied perpendicularly in the midpalatal suture on a single point just behind the superior incisors.

In order to deliver to the tissue, the equivalent energy (J) of 18 J for both laser and LED equipment, considering that the light sources presented spots and output values very discrepant from each other, spatial average energy fluence (SAEF) was calculated (18 J/cm^2) and used as our administered dosage. The area used for the calculation of SAEF was 1 cm^2, instead of the spot area, regarding the scattering in the tissue and the Gaussian distribution. Furthermore, it was requested to the manufacturer that both the laser and LED equipment used in this research be calibrated considering this area of 1 cm^2 in the calculation for its supply of the energy dosage, therefore providing a fairer comparison between the equipment. The total dose used for the 7-day groups was 54 J/cm^2 and for the 14-day groups 108 J/cm^2 (Table 2).

Table 1 Protocol of expansion, laser, and LED irradiations

Groups	Treatment	Days 1, 3, 5	Days 8, 10, 12
1	Control	–	–
2	Expansion	–	–
3	Expansion + laser 7 days	–	–
4	Expansion + LED 7 days	LED	–
5	Expansion 14 days	–	–
6	Expansion + laser 14 days 1st	Laser	–
7	Expansion + LED 14 days 1st	LED	–
8	Expansion + laser 14 days 2nd	Laser	Laser
9	Expansion + LED 14 days 2nd	LED	LED

At every irradiation session, the animals were kept under anesthesia using the same protocol previously described but using only one third of the dose as irradiation demanded less time than when installing the device. A mouth opener was used during irradiation. The animals were killed at the end of each experimental period (7 or 14 days) with an overdose of general anesthesia.

The maxilla of all animals was dissected and sliced transversally in two halves. The inferior half of the maxilla was stored in liquid nitrogen and used for Raman spectroscopy. Liquid nitrogen was used to minimize bacterial growth and because chemical fixation is not advisable due to fluorescence emissions from the fixative substances. The area of the

Table 2 Laser and LED parameters

Parameters	Laser	LED
Wavelength (nm)	780	850 ± 10
SAEF (J/cm^2)	18	18
Energy (J)	18	18
Output (mW)	70	150
Output (W)	0.07	0.15
Illuminated area in the tissue (cm^2)	1	1
Mode	CW	CW
Application	Contact	Contact
Spot (cm^2)	0.04	0.5
Energy density (J/cm^2)	450	36
Power density (W/cm^2)	1.75	0.3
Exposure time per session (s)	257	120

midpalatal suture evaluated corresponded to the same point in the midpalatal suture where LED and laser light were applied. A baseline Raman spectrum of nontreated bone (cortical bone) was also produced as a control.

The experiment was carried out in the Laboratory of Biomolecular Spectroscopy of the Center of Biophotonics of the School of Dentistry of the Federal University of Bahia (Salvador, BA, Brazil). A dispersive Andor Shamrock SR-303i Raman spectrophotometer (Andor Technology, Belfast, Northern Ireland) was used. The equipment used a stabilized diode laser (λ785 nm, B&WTEK, Newark, DE, USA) with an output of 500 mW obtained through an optic fiber ("Raman Probe"). This probe was positioned in contact with the samples, which were analyzed in vitro, and data was collected.

The useful spectral bandwidth ranged from 200 to 1800 cm^{-1}, and the luminous signal detection scattered by the sample was accomplished through a CCD iDUS (Andor Technology, Belfast, Northern Ireland) back thinned, deep depletion 1024 × 128 pixel camera, cooled by a thermoelectric cooler, reaching −70 °C temperature in 5 min counting from the start of the spectrometer operation. The acquisition and storage of the spectrums was achieved by a microcomputer using Andor Solis® software (Andor Technology, Belfast, Northern Ireland), which controls via USB connection the exposure time of the detector and the number of acquisitions of the samples and promotes the storage of the spectrums for posterior analysis and interpretation. The exposure time for obtaining the spectrums was 20 s accumulated 5 times, with an output of 500 mW. This acquisition time and output did not cause damage to the samples. Five readings of each sample were obtained in order to calculate the mean and standard deviation values for all samples.

The spectrometer's wavelength was calibrated by the manufacturer. Before data collection, naphthalene spectrum was collected and its band positions (Raman displacement) were compared to those reported in the literature, in the 500- and 1700-cm^{-1} spectral region which is the region of interest for Raman spectrometry used for biological materials analysis (fingerprint region). After calibration of the Raman displacement and acquisition of the spectrums in vitro, data was preprocessed and stored for posterior statistical analysis. The preprocessing consisted of the removal of the background fluorescence, which corresponds to the low-frequency spectral components (fluorescence) and posterior subtraction of the original data revealing the high-frequency spectral components (Raman). The spectrums had their intensity maintained. This preprocessing was obtained using MatLab 4.0® software (Mathworks, MA, USA).

The Raman spectrum of bone presents prominent vibrational bands related to tissue composition (mineral and organic components). The medium spectrum and medium value of peak intensity of phosphate (~960 cm^{-1}) and CH groups of lipids and protein (~1450 cm^{-1}) levels were determined by the difference between the maximum and minimum intensity measured from each. These intensities are related to the concentration of CHA (hydroxyapatite) and organic components (lipids and proteins (collagen) of the bone. All data collected was submitted to statistical analysis using ANOVA and Student's *t* tests.

The superior half of the maxilla was stored in 10% formaldehyde for 3 days and processed in the Laboratory of Surgical Pathology of the School of Dentistry of the Federal University of Bahia (Salvador, BA, Brazil) for histological analysis. The specimens were decalcified in 5% formic acid for 24 to 48 h and were routinely processed to paraffin, and transverse 4-μm-thick sections were obtained and stained with hematoxylin and eosin and picrosirus (collagen). The area of the midpalatal suture evaluated corresponded to the same point in the midpalatal suture where LED and laser light were applied. A semiquantitative method was used for the histological evaluation using the criteria described in Table 3. This analysis was carried out by an experienced pathologist in a blind manner. Data was submitted to statistical analysis using ANOVA and Fisher's exact test.

Table 3 Criteria used for the histological semiquantitative analysis

Criteria	Absence	Discrete	Moderate	Intense
Inflammatory process	−	Presence of <25% of mononuclear cells	Presence of 25–50% of mononuclear cells	Presence of 50% of mononuclear cells
Collagen fibers	−	Presence of <25% of collagen	Presence of 25–50% of collagen	Presence of 50% of collagen
Osteoblastic activity	−	Presence of <25% of osteoblastic activity	Presence of 25–50% of osteoblastic activity	Presence of 50% of osteoblastic activity
Osteoclastic activity	−	Presence of <25% of osteoclastic activity	Presence of 25–50% of osteoclastic activity	Presence of 50% of osteoclastic activity

Results

Near-infrared Raman spectroscopy

Midpalatal suture

The intensity of the Raman shift at ~1450 cm^{-1} (relative to the matrix collagen) represents the presence of collagen. There was significant statistical difference in the collagen peaks when comparing all groups on the 7th day. Comparison of the control group to all the other groups (2, 3, and 4) showed significant statistical difference in all cases. When only treated groups 2, 3, and 4 were analyzed, no statistical difference was observed. The paired Student's *t* test also showed no statistical difference, even though group 4 presented the highest mean peak value (729.1 ± 268.6) (Table 4).

In the 14-day groups, the intensity of the Raman shift at ~1450 cm^{-1} also showed significant statistical difference regarding the collagen peaks. Paired Student's *t* test comparing the control group with groups 5, 6, 7, 8 and 9 also showed significant differences. The control group showed the lowest mean collagen peak (3310 ± 727). When comparing groups 5, 6, 7, 8, and 9, significant statistical difference was found also. Student's *t* test demonstrated significant statistical difference between groups 5 and 7 and between groups 7 and 9. Time also influenced the outcome of the procedure in some groups as the comparison between the 7- and 14-day groups showed significant difference only between groups 4 and 7 (Table 4).

The intensity of the Raman shift at ~960 cm^{-1} (relative to mineral phosphate) is directly related to the concentration/incorporation of CHA by the bone. Higher intensities represent higher concentrations of CHA. There was a statistically significant difference in the CHA peaks between all 7-day groups (1, 2, 3, and 4) in the midpalatal suture, with group 4 presenting the highest mean peak value (4614.4 ± 1770.4). Significant statistical difference was also observed when comparing only treated groups 2, 3, and 4 with group 4 also having the highest mean peak value. When comparing the control group to each of treatment groups 2, 3, or 4 individually, significant statistical difference was found for all comparisons. The control group showed the lowest mean peak value (1603.7 ± 261.3). Group 2 compared individually to irradiated groups 3 and 4 also presented significant statistical difference (Table 5).

When comparing the control group to all 14-day groups, significant statistical difference was found. Individual comparison between the control group and all other 14-day groups also found significant statistical difference. Comparison between groups 5, 6, 7, 8, and 9 also found significant statistical difference. Student's *t* test showed significant statistical difference between groups 5 and 8, groups 5 and 9, groups 7 and 9, and groups 8 and 9. Comparison of groups along the times also showed significant difference between groups 4 and 7 and between groups 4 and 9 (Table 5).

Cortical bone

There was significant statistical difference when comparing the collagen peaks between all 7-day groups (1, 2, 3, and 4) in the cortical bone. The control group showed the lowest mean peak value (351.3 ± 997). When only the treated groups were compared, no statistical difference was found. However, group 4 presented the lowest mean peak value (552 ± 168) (Table 6). Significant statistical difference was found between the control group and all the other 14-day groups. When comparing the control group with the treated groups (5, 6, 7, 8, and 9), statistical difference was also found. When comparing the treated groups, a significant difference was observed between groups 5 and 6, 5 and 7, 6 and 9, and 7 and 9 (Table 6).

Significant statistical difference was found when comparing the CHA peaks in the cortical bone between groups 1, 2, 3, and 4. The control group showed the lowest mean peak (19,920 ± 6196). No statistical difference was found when comparing only treated groups 2, 3, and 4. However, group 4 showed the highest mean peak value (3073.1 ± 261.3) (Table 7).

Table 4 Mean values ± SD of the Raman peaks for collagen (~1450 cm^{-1}) in the midpalatal suture of all groups ($n = 5$)

Group	Treatment	Means ± SD
1	Control (a)	3310 ± 727 (b, c, d, e, f, g, h, i)
2	Expansion (b)	7784 ± 2004 (a)
3	Expansion + laser 7 days (c)	7291 ± 2686 (a)
4	Expansion + LED 7 days (d)	8410 ± 1777 (a, g)
5	Expansion 14 days (e)	7589 ± 1032 (a, g)
6	Expansion + laser 14 days 1st (f)	6221 ± 2425 (a)
7	Expansion + LED 14 days 1st (g)	5025 ± 592 (a, d, e, i)
8	Expansion + laser 14 days 2nd (h)	6366 ± 3392 (a)
9	Expansion + LED 14 days 2nd (i)	7445 ± 2186 (a, g)

Lowercase letters indicate that the value is significantly different from the value of the group with the same letter

Table 5 Mean values ± SD of the Raman peaks for CHA (~960 cm^{-1}) in the midpalatal suture of all groups ($n = 5$)

Group	Treatment	Means ± SD
1	Control (a)	16,037 ± 2613 (b, c, d, e, f, g, h, i)
2	Expansion (b)	27,048 ± 15,834 (a, c, d)
3	Expansion + laser 7 days (c)	34,479 ± 19,701 (a, b)
4	Expansion + LED 7 days (d)	46,144 ± 17,704 (a, b, g, i)
5	Expansion 14 days (e)	23,433 ± 4752 (a, h, i)
6	Expansion + laser 14 days 1st (f)	31,298 ± 14,526 (a)
7	Expansion + LED 14 days 1st (g)	26,415 ± 7072 (a, d, h)
8	Expansion + laser 14 days 2nd (h)	35,910 ± 1504 (a, e, g, i)
9	Expansion + LED 14 days 2nd (i)	27,938.8 ± 5490 (a, d, e, h)

Lowercase letters indicate that the value is significantly different from the value of the group with the same letter

Comparison of the control group with all 14-day groups demonstrated significant difference between them. However, when comparing only the treated groups (5, 6, 7, 8, and 9), no statistical difference was found. Group 8 showed the highest mean peak (35.121 ± 26.724) (Table 7).

Histological and histomorphometric analyses

Inflammation was observed in group 1 (Fig. 2a) and in all the other groups. Chronic inflammation was observed in groups 4 (Fig. 2b) and 6 (Fig. 2c). Fisher's exact test ($p \leq 0.005$) indicated significant difference between group 5 and all the other groups. Group 5 presented discrete inflammation in 100% of the specimens analyzed. When comparing group 5 to groups 1, 2, 6, 8, and 9, there was also significant difference. Comparing groups 5 and 2 also showed significant difference being similar to that observed between groups 4 and 7 (Fig. 2d).

Regarding collagen deposition, discrete presence of collagen was observed in group 2 (Fig. 3a), while groups 6 and 7 presented moderate (Fig. 3b) and intense (Fig. 3c) collagen deposition, respectively. Statistical difference was found when comparing group 2 to all the other groups, except group 5. Significant difference was also observed between groups 2 and 1, 2 and 3, and 2 and 4, 6, 7, 8, and 9 (Fig. 3d).

Osteoblastic activity was evaluated between groups. The untreated expansion group showed osteoblastic activity and giant cells in activity at 7 (Fig. 4a) and 14 days (Fig. 4b), respectively. On day 14, osteoblastic activity was observed in the expansion group treated with LED (Fig. 4c). On day 7, the untreated expansion group demonstrated giant multinuclear cells and severe chronic inflammation, while the laser-treated group showed irregular osteocytes, numerous giant multinuclear cells, and chronic inflammation, and the LED-treated group showed osteocytes and osteoclastic cells. Significant differences were found between groups 4 and 1 and 2, 4 and 3, 4 and 5, and 4 and 8. Groups 5 and 6 were also significantly different. Osteoclastic activity also showed significant difference when comparing group 2 with groups 1, 6, and 8 as well as between groups 5, 6, and 8 (Fig. 5a–d).

Discussion

It has been suggested that light irradiation increases both the number and activity of osteoblasts [6, 20] and leads to a higher

Table 6 Mean values ± SD of the Raman peaks for collagen (~1450 cm^{-1}) in the cortical bone of all groups ($n = 5$)

Group	Treatment	Means ± SD
1	Control (a)	3513 ± 997 (b, c, d, e, f, g, h, i)
2	Expansion (b)	6503 ± 2559 (a)
3	Expansion + laser 7 days (c)	7030 ± 3573 (a, f)
4	Expansion + LED 7 days (d)	5520 ± 1680 (a, i)
5	Expansion 14 days (e)	6699 ± 1376 (a, f, g)
6	Expansion + laser 14 days 1st (f)	4742 ± 801 (a, c, e, i)
7	Expansion + LED 14 days 1st (g)	5147 ± 515 (a, e, i)
8	Expansion + laser 14 days 2nd (h)	5875 ± 3158 (a)
9	Expansion + LED 14 days 2nd (i)	6729 ± 1931 (a, d, f, g)

Lowercase letters indicate that the value is significantly different from the value of the group with the same letter

Table 7 Mean values ± SD of the Raman peaks for CHA (~960 cm^{-1}) in the cortical bone of all groups ($n = 5$)

Group	Treatment	Means ± SD
1	Control (a)	19,920 ± 6196 (b, c, d, e, f, g, h, i)
2	Expansion (b)	24,101 ± 11,325 (a)
3	Expansion + laser 7 days (c)	28,682 ± 12,822 (a)
4	Expansion + LED 7 days (d)	30,731 ± 2613 (a)
5	Expansion 14 days (e)	21,145 ± 6110 (a)
6	Expansion + laser 14 days 1st (f)	29,868 ± 10,725 (a)
7	Expansion + LED 14 days 1st (g)	31,414 ± 10,599 (a)
8	Expansion + laser 14 days 2nd (h)	35,121 ± 26,724 (a)
9	Expansion + LED 14 days 2nd (i)	29,333 ± 6416 (a)

Lowercase letters indicate that the value is significantly different from the value of the group with the same letter

calcium deposition rate and bone repair in comparison to non-irradiated subjects [21].

Previous studies using Raman spectral analysis have also demonstrated higher deposition of CHA in laser- or LED-

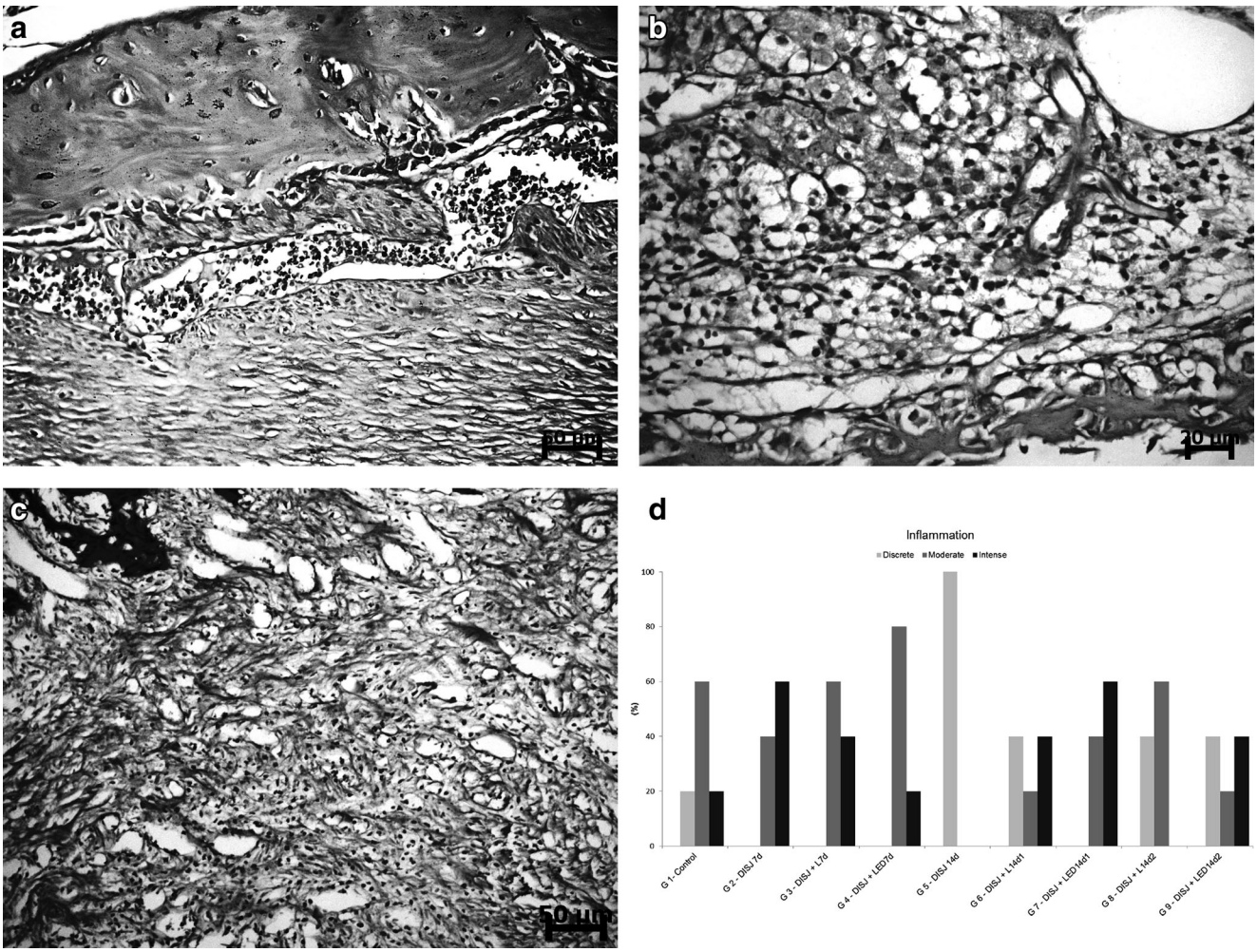

Fig. 2 **a** Photomicrograph of a specimen of the control group showing the inflammatory reaction. Photomicrograph of a specimen showing the presence of chronic inflammation on group expansion + LED 7 days (**b**) and on group expansion + laser 14 days (**c**). Photomicrograph of a specimen showing the presence of chronic inflammation. **d** Summary of the histomorphometry regarding the inflammatory reaction observed in the present investigation

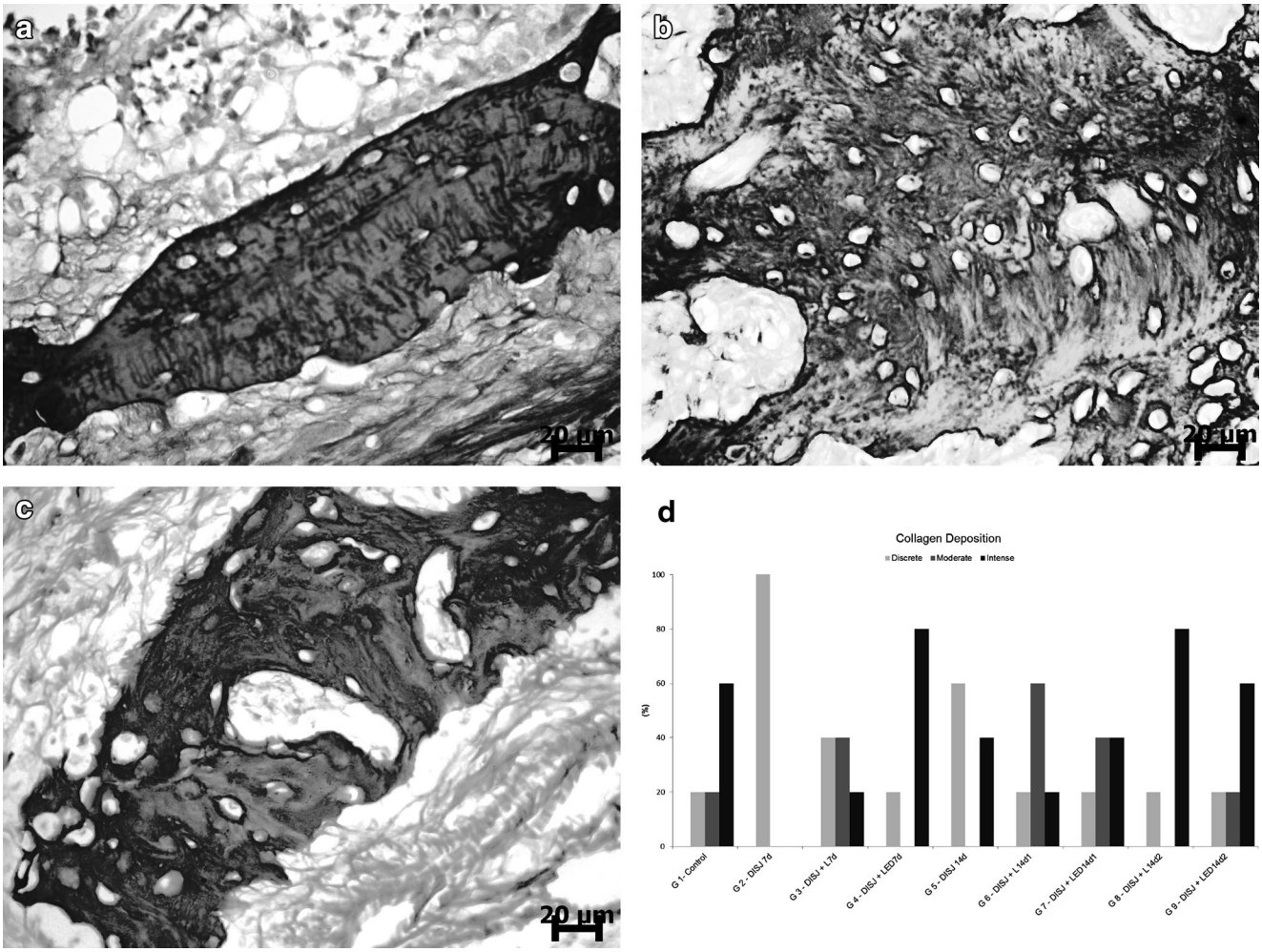

Fig. 3 **a** Photomicrograph of a specimen of the group expansion 7 days showing a discrete presence of collagen. On group expansion + laser 14 days, a moderate presence of collagen was seen (**b**). On the other hand, the group expansion + LED 14 days showed intense collagen expression (**c**). **d** Summary of the histomorphometry regarding the collagen expression observed in the present investigation

irradiated bone defects. The authors suggested that the presence of more mature osteoblasts with improved ability of secreting CHA seen on irradiated animals differed from the non-irradiated groups in which cell proliferation was still occurring [22]. Increased amounts of CHA may be positively correlated to bone mineral density, for higher calcium intakes result in an increase in bone mineral density [23]. Deposition of CHA represents bone maturation [22].

Osteogenesis, however, is a result of a balance between osteoblastic and osteoclastic activities, and in various circumstances, bone formation is accompanied by bone resorption [24] as observed in the midpalatal suture in the present study, as the osteoclastic activity observed was compatible to the variations observed in the peaks of CHA.

Previous reports suggested that the use of laser therapy associated to midpalatal suture expansion is effective for bone regeneration, accelerating mineral apposition and causing a quicker repair process in the area [1, 7]. A previous study has demonstrated that a single laser application after rapid expansion induced changes in the osteoblastic activity and that this lasted for a long period of time [25]. Laser irradiation could potentially recruit osteoblasts and/or their maturation throughout the bone borders of the midpalatal suture [1]. To the best of our knowledge, there is only one study in the literature that accesses histologically the use of LED combined with the expansion of the midpalatal suture [26], which makes the discussion of our results very difficult in this aspect.

The protocol used in the present study using LED light is similar to the laser protocol used by our team in previous studies and also in this research. Recent researches have indicated that LED, operating in several wavelengths, has beneficial effects and similar mechanisms as the ones observed when laser is used [10], in vitro and in vivo, in both normal and pathologic conditions [4, 13]. Experimentation using LED light treatments at various wavelengths has shown to significantly increase cell growth in a variety of cell lines, including fibroblasts and osteoblasts [4]. LED-irradiated bone (mostly irradiated with IR wavelengths) seems to increase

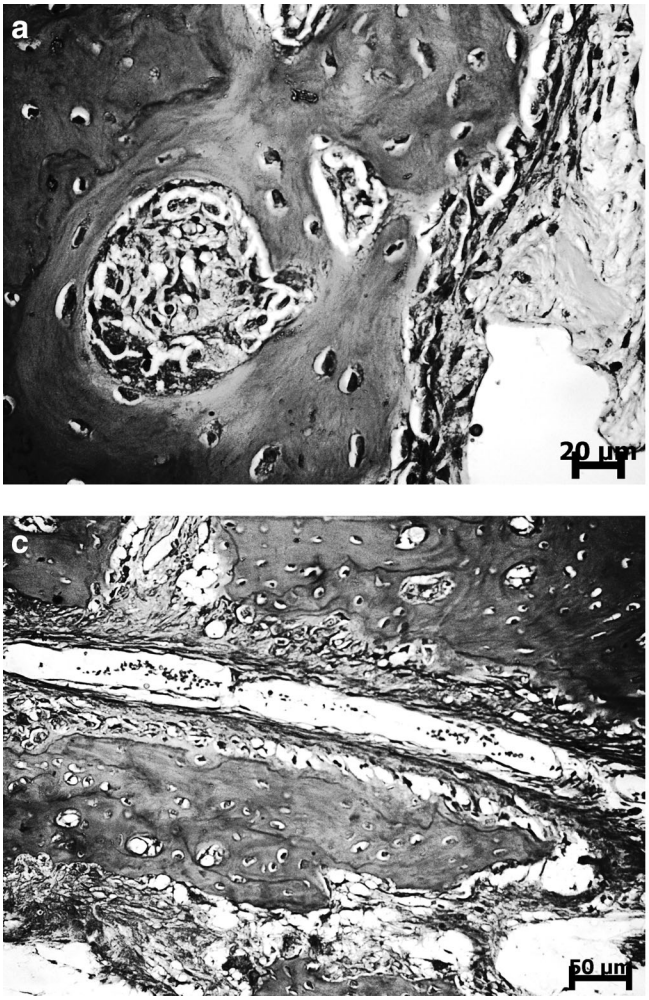

Fig. 4 **a** Photomicrograph of a specimen of the group expansion 7 days evidencing osteoblastic activity. **b** Photomicrograph of a specimen of the group expansion 14 days evidencing the presence of giant cells. **c** Photomicrograph of a specimen of the group expansion + LED 14 days evidencing osteoblastic activity. **d** Summary of the histomorphometry regarding the osteoblastic activity observed in the present investigation

osteoblastic proliferation, collagen deposition, and bone neoformation [6] and improves the quality of the newly formed bone due to increased deposition of CHA as seen in other Raman studies when compared to nonirradiated bone [10]. This also corroborates our findings.

In this study, collagen deposition was evaluated in the midpalatal suture through Raman spectroscopy. The results evidenced that in all groups, on both 7 and 14 days, submitted to rapid maxillary expansion and irradiated or not with laser or LED presented a significant increase of collagen deposition when compared to the untreated group. The control group was not submitted to any treatment and therefore had no stimulation to accelerate collagen deposition and posterior mineralization beyond what is normally expected in an animal under growth [27]. On the other hand, all the expansion groups showed high peaks of collagen, which is expected after the expansion procedure, as a suture under tension stimulates extracellular matrix deposition [28].

For comparison purposes, as the bone rated in the sutures was newly formed due to the direct effect of active orthopedic disjunction, the cortical bone of the mice's jaw used in this study served as a reference in the analysis of Raman spectroscopy.

Regarding collagen in the cortical bone, the control group had lower peak average than all the treated groups much on the 7th and 14th days. However, in this case, the less amount of collagen is related to the fact that the cortical bone is more mature, compatible with a lower intensity of collagen [29]. As observed in the suture, no difference was found when the groups treated at 7 days were compared. On day 14, among the groups treated on the 14th day, a higher average for group 9 than for group 7 was also found, indicating that when irradiation is extended over 14 days, there is greater stimulation of fibroblasts and the production of collagen fibers intensifies.

No difference was found when the groups treated at 7 days together were compared. Among the groups treated at 14 days, it was also found that there is a higher average for group 9 when compared to group 7, indicating that when irradiation is extended over 14 days, there is greater stimulation of fibroblasts and the production of collagen fibers intensifies.

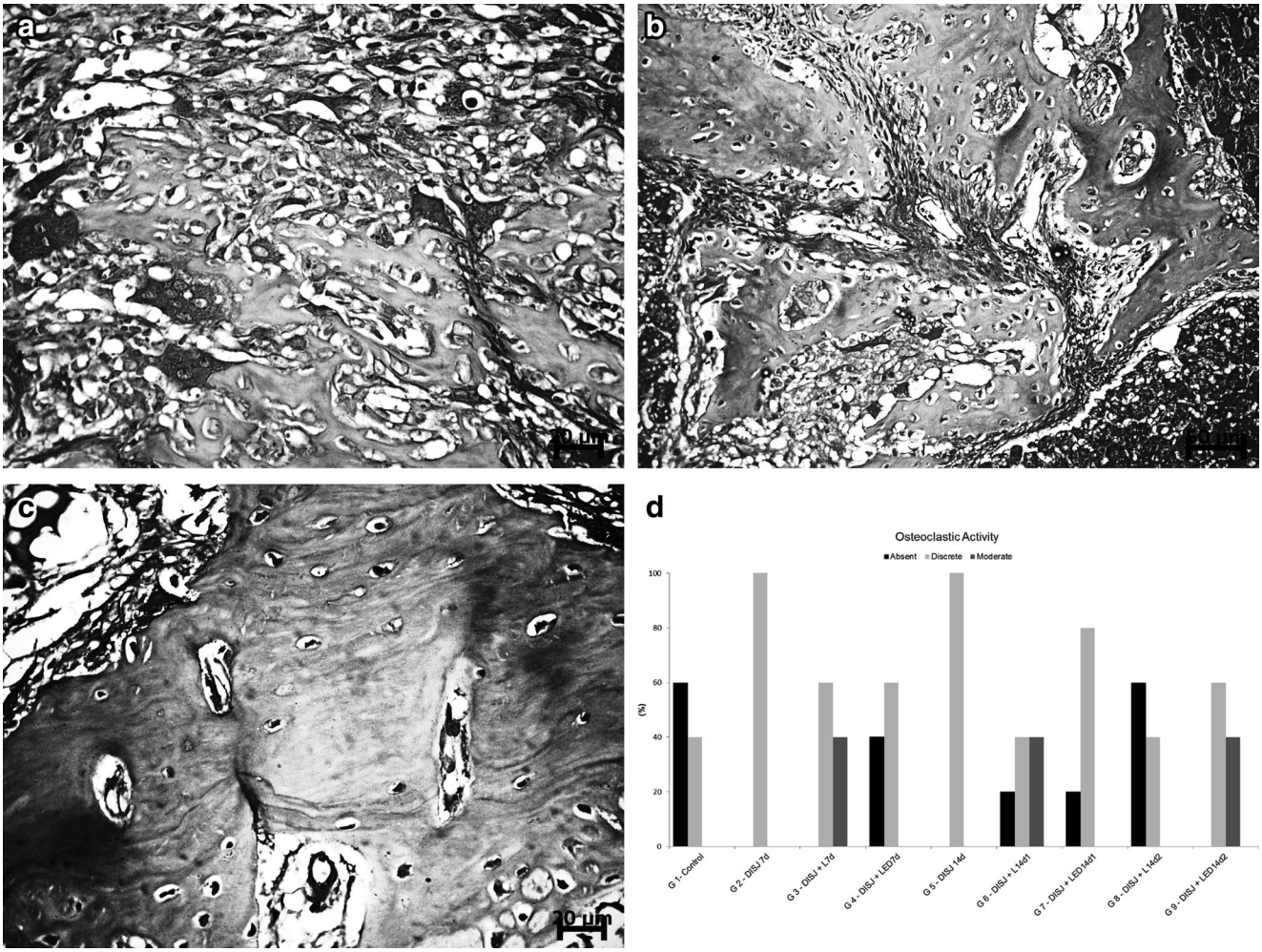

Fig. 5 **a** Photomicrograph of a specimen of the group expansion 7 days showing giant multinuclear cells and severe chronic inflammation. **b** Photomicrograph of a specimen of the group expansion laser 7 days evidencing the presence of irregular osteocytes, numerous giant multinuclear cells, and chronic inflammation. **c** Photomicrograph of a specimen of the group expansion laser 7 days showing the presence of osteocytes and osteoclasts. **d** Summary of the histomorphometry regarding the osteoclastic activity observed in the present investigation

Regarding the deposition of hydroxyapatite in cortical bone, statistically significant differences were found in the control group when compared to the other treated groups at 7 and 14 days. These results are similar to those obtained when assessing the suture where the control group also had lower peak average. It should be highlighted that the maxillary cortical bone was not a direct site of the orthopedic effect of disjunction as the machine used was not attached to the palatal mucosa. On the other hand, all groups submitted to the disjunction had higher HA mean peaks with no statistical difference among them. When opening the suture, dissipated forces may have caused an indirect effect in the area culminating in a more intense deposition of hydroxyapatite in the treated groups than in the control group.

In addition, the recruitment of osteoblastic cells to the area of the disjunction may have increased the cortical bone mineral deposition in this region. Another possible contributing factor was that the spot area of both light devices was larger than the area of the sutures resulting in laser and LED effects in the cortical bone adjacent to the suture. It should be noted that polymerization does not occur only in the area of the incident beam but also in the area equally distributed around the three-dimensional shape. According to the principles of diffusion, transmission, and reflection of the laser beam on the tissue, depending on the wavelength, laser efficiency extends about 1 cm in diameter with its center being the point of incidence [30].

The use of either laser or LED is known to stimulate the proliferation of fibroblasts, which are major secretors of collagen and intensify collagen deposition [10]. However, our results showed no statistical difference on the 7th day with regard to collagen deposition between groups 2, 3, and 4. Although no statistical difference was found, LED irradiation caused a higher peak value for organic contents. According to previous studies, LED-irradiated bone shows a high quantity of collagen that is possibly associated with an increased

collagen deposition by fibroblasts stimulated by LED light similar to that observed when using laser light [10, 22]. With progression of the repair, on the 14th day, significant statistical difference was observed between groups 5 and 7. The lowest mean peak ~1450 cm^{-1} detected in group 7 may be related to an increased deposition of hydroxyapatite observed in all LED-irradiated groups when compared to the groups treated only with expansion. This finding is fully aligned with a previous report that mentioned that as bone repair advances, the concentration of collagen is reduced due to an increase in the mineral-organic matrix ratio [29]. On the other hand, when continuously irradiating throughout the 2-week period, the LED groups showed increased collagen deposition, being higher in group 9 than in group 7. The fact that group 9 was irradiated throughout the 14 days could possibly have resulted in a more intense collagen deposition than in group 7 where light stimulation was removed after 7 days. This finding may be justified by the results of a previous study that suggested that continuous light irradiation beyond the initial stages after rapid maxillary expansion could maintain the regenerative activity in the suture [1].

Interestingly, the laser-irradiated groups were not statistically different regarding collagen deposition between them or in comparison to the LED-irradiated ones. Although no statistical difference was also found between groups treated only with expansion (groups 2 and 5), it may be noticed that the laser-irradiated groups always showed higher peaks of hydroxyapatite. This may be indicative of an acceleration of the mineralization. It is important to notice that the histological results indicated that the collagen deposition in either the laser or LED groups varied from moderate to intense. This may be indicative of both increased proliferation and secretion of fibroblasts caused by light irradiation as reported previously [6, 10, 14, 22].

The results of the present study showed that the level of CHA in the midpalatal suture was affected by all treatments used as the intensity of the ~960-cm^{-1} peak was significantly lower than all the other. Interestingly, the groups submitted to expansion associated or not to laser or LED light (groups 2, 3, and 4) archived different intensities of the CHA peak at day 7, and the use of laser (group 3) or LED (group 4) after expansion resulted in a higher mineralization of the midpalatal suture when compared to the group in which only the expansion procedure was executed (group 2).

Despite that no significant difference was found between the two irradiation protocols, the use of LED light resulted in higher peak of CHA, therefore indicating a greater mineralization of the area. This corroborates our histological findings in which it was observed that, on the 7th day, the use of LED resulted in an intense osteoblastic activity in all specimens. These early findings corroborate with a similar study using LED light (618 nm) at an energy density of 20 mW/cm^2 for 20 min over a period of ten consecutive days that observed histologically higher values of new bone formation area, number of osteoblasts, number of osteoclasts, and number of vessels in the expanding suture area in the experimental group [26]. Surprisingly, on the 14th day, no difference was found between groups 5, 6, and 7. It is important to observe that when irradiation was carried out only in the 1st week (groups 6 and 7), a reduction in the concentration of CHA was observed, with mineralization at the end of the experimental period equivalent to the group treated only with expansion (group 5). This result is in agreement with another research that used laser and found that extending the irradiation period beyond the initial stages of the expansion procedure could maintain and accelerate bone formation [1].

A very interesting finding of the present study was that, when irradiation was performed throughout 2 weeks, irradiated groups 8 and 9 showed a more mineralized tissue when compared to expansion-only group 5. Therefore, it could imply that maintaining the irradiation with either laser or LED during 2 weeks may stimulate CHA deposition. This finding is also in agreement with our histological results where a more intense osteoblastic activity was seen on the 14th day and therefore related to bone formation and mineralization.

Raman spectroscopy used to analyze alterations in both mineral and organic bone components is considered a gold standard analysis [5]. In this study, Raman spectral analysis and histological findings indicated that both laser and LED phototherapy increased collagen deposition possibly due to the histologic finding of increased osteoblastic activity. The increased deposition of CHA in the midpalatal suture after expansion is indicative of an acceleration of bone maturation in the area. This is important as a more mature bone reduces the retention period and consequently reduces orthodontic treatment time. Therefore, during expansion, a few visits to the dental office for phototherapy could lead to a faster response and be beneficial for the patient. LED radiation has the advantage of a lower cost compared to laser and it can safely be applied to body surfaces [27]. Even though we observed favorable results when using different light sources, our knowledge of bone neoformation and light interactions is still limited. More studies, specially using LED light, are necessary.

The results of the present study are indicative that LED irradiation associated to rapid maxillary expansion improves bone repair and could be an alternative to the use of laser in accelerating bone formation in the midpalatal suture.

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Compliance with ethical standards

Conflict of interest The authors received a grant from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), a

government research agency, but have full control of all primary data and agree to allow the journal to review their data if requested.

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