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Effect of low-level laser therapy on the healing process after tooth replantation: a histomorphometrical and immunohistochemical analysis

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Abstract – Success of tooth replantation is limited because part of the replanted tooth is lost because of progressive root resorption. This study used histomorphometry and immunohistochemistry to evaluate the effect of low-level laser therapy (LLLT) on the healing process of rat teeth replanted after different extra-oral periods, simulating immediate and delayed replantation. Sixty Wistar rats (*Rattus norvegicus albinus*) had their maxillary right incisors extracted and randomly assigned to six groups ($n = 10$): C4, C30 and C45, in which the teeth were replanted 4 min (immediate), 30 min (delayed) and 45 min (delayed) after extraction, respectively, and L4, L30 and L45, in which the teeth were replanted after the same extra-alveolar times, but the root surfaces and the alveolar wounds were irradiated with a gallium–aluminum–arsenate (GaAlAs) diode laser before replantation. The animals were sacrificed after 60 days. The anatomic pieces containing the replanted teeth were obtained and processed for either histomorphometrical analysis under optical microscopy or immunohistochemical expression of receptor activator of nuclear factor Kappa-B (RANK), and its ligand (RANKL), osteoprotegerin (OPG) and tartrate-resistant acid phosphatase (TRAP) proteins. Areas of external replacement and inflammatory root resorption were observed in all groups, without statistically significant differences ($P > 0.05$). Ankylosis was more frequent in L30 than in C30 ($P < 0.05$). RANKL immunostaining predominated over RANK and OPG immunostaining in both groups with immediate tooth replantation ($P < 0.05$). For the 45-min extra-alveolar time, however, there was greater evidence of RANK immunostaining compared to RANKL for both control and laser-treated groups ($P < 0.05$). Positive TRAP immunostaining predominated in L4 and L30 ($P < 0.05$). In conclusion, under the tested conditions, the treatment of the root surface and the alveolar wound with LLLT did not improve the healing process after immediate and delayed tooth replantation in rats.

Early tooth loss because of trauma compromises the integrity of the natural dentition, and thus, all efforts must be directed to the immediate replantation of traumatically avulsed teeth in order to offer favorable conditions for the healing process (1–3). The treatment of root surfaces has been extensively investigated in an attempt to aid the periodontal ligament (PDL) repair and increase the chances of success in replantation procedures (4–10). However, the prognosis of most cases is uncertain because of the potential occurrence of external root resorption (3, 10).

In recent years, the possible benefits of low-level laser therapy (LLLT) on alveolar wound repair after tooth replantation have been considered based on a series of biologic effects that culminate in process known as photobiostimulation. Laser energy is absorbed by inter- and intra-cellular targets, producing a secondary stimulation of tissue healing mechanisms with anti-inflammatory and analgesic effects (11).

Immunohistochemistry is a methodological tool that can be used to investigate the action of LLLT on the mechanisms involved in cellular repair in tooth replantation, and help elucidating aspects that so far have more often been investigated histomorphometrically (5–9).

The proteins belonging to the tumor necrosis factor (TNF) superfamily – osteoprotegerin (OPG) (12), receptor activator of nuclear factor Kappa-B (RANK) (13) and its ligand (RANKL) (14) – are intimately linked to the dynamics of bone resorption and remodeling processes. The balance between the expressions of the components of the RANK/RANKL/OPG system provides important information about bone metabolism (15) because these

proteins participate in bone tissue dynamics and homeostasis. Tartrate-resistant acid phosphatase (TRAP), an isoenzyme of acid phosphatase that is found mainly in the bone tissue and some blood cells, also has an important role in resorptive processes by acting as a marker of the enzyme that demonstrates the osteoclastic activity (16).

This study used histomorphometry and immunohistochemistry to evaluate the effect of LLLT on the healing of rat teeth replanted after different periods, simulating immediate and delayed replantation.

Material and methods

The research protocol was approved by the Animal Research Ethics Committee of the School of Dentistry Araçatuba (São Paulo State University, Brazil; Protocol No. 19/06).

Sixty male Wistar rats (*Rattus norvegicus*, *albinus*) weighing 250–300 g were used. The animals were fed with ground solid ration (Guabi Nutrilabor, Mogiana Alimentos SA, Campinas, SP, Brazil) and water *ad libitum*, except for the preoperative 12 h. Before the procedures, the animals received an intramuscular injection of xylazine hydrochloride (Dopaser, Laboratório Calier do Brasil. Ltda, Osasco, SP, Brazil; 0.6 mg per 100 g body weight) for muscular relaxation and were anesthetized with ketamine hydrochloride (Dopalen; AgriBrands do Brasil Ltda, Paulinea, SP, Brazil; 0.7 mg per 100 g body weight).

The maxillary right incisors of all animals were extracted in a non-traumatic manner and were randomly assigned to 6 groups of 10 specimens each, as follows:

1 Group C4 (4-min extra-alveolar time – control – immediate replantation): the teeth were held by their crowns, fixed on a red wax plate and kept dry at room temperature for 4 min. Asepsis of the anterior maxilla with 1% iodine polyvinylpyrrolidone (Riodeine; Indústria Farmacêutica Rioquímica Ltda., São José do Rio Preto, SP, Brazil) was performed, the sockets were gently irrigated with saline and the teeth were replanted.
2 Group C30 (30-min extra-alveolar time – control – delayed replantation): the teeth were stored in saline for 30 min and then the dental papilla and the enamel organ of each tooth were removed with a #15 scalpel blade (Wuxi Xinda Medical Device Co Ltd., Zhanging, Xishan, China) and the pulp tissue was extirpated through a retrograde via with a slightly curved #35 Hedström file (25 mm; Sybron Kerr Corporation, Orange, CA, USA). Root canals were irrigated with saline (Ariston Ind. Quím. e Farm. Ltda, São Paulo, SP, Brazil), dried with absorbent paper points and filled with a calcium hydroxide (Biodinâmica Química e Farmacêutica Ltda., Ibiporã, PR, Brazil) and propyleneglycol (Manipullis, Araçatuba, SP, Brazil) paste using a 28 × 8 mm hypodermic needle coupled to an insulin syringe (Injex Indústrias Cirúrgicas Ltda., Ourinhos, SP, Brazil). Asepsis of the anterior maxilla with 1% iodine polyvinylpyrrolidone (Riodeine; Indústria Farmacêutica Rioquímica Ltda.) was performed, and the teeth were replanted into their respective sockets.
3 Group C45 (45-min extra-alveolar time – control – delayed replantation): the teeth received the same treatment described for C30, expect for the extra-alveolar time in saline storage, which was 45 min in this group.
4 Group L4 (4-min extra-alveolar time – laser – immediate replantation): the palatal root surfaces of the teeth were treated with a gallium-aluminum-arsenate (GaAlAs) continuous-wave (CW) diode laser emitted at 660 nm wavelength (visible light), 30 mW output power, 4 J total energy, and 57.14 J cm^{-2} energy density (fluency). A single application was carried out in the continuous emission mode with short scanning movements along the long axis of the root within a total irradiation time of 2 min and 13 s. The alveolar wounds were treated with an infrared GaAlAs CW diode laser with 830 nm wavelength, 40 mW output power, 4 J total energy, and 57.14 J cm^{-2} energy density (fluency). Irradiation was carried out in a single, punctual application in the contact mode at the middle third of the palatal surface of alveolar wound within an irradiation length of 1 min and 40 s. Knowing the output power of each laser (30 and 40 mW, for visible and infrared laser, respectively) and the energy used in the study (4 J for both lasers), the following equation was used to calculate the exposure time to be set for each laser: energy (J) = power (W) × exposure time (s). Asepsis of the anterior maxilla with 1% iodine polyvinylpyrrolidone (Riodeine; Indústria Farmacêutica Rioquímica Ltda, São José do Rio Preto, SP, Brazil) was performed, and the teeth were replanted into their respective sockets.
5 Group L30 (30-min extra-alveolar time – laser – delayed replantation): After storage in saline for 30 min, the teeth were treated in the same way as described in the group C30 up to root canal filling with the calcium hydroxide and propylene glycol paste. Then, the palatal root surfaces and the alveolar wounds were treated with the lasers in the same way as described in the group L4, and the teeth were replanted into their respective sockets.
6 Group L45 (45-min extra-alveolar time – laser – delayed replantation): After storage in saline for 45 min, the teeth were treated in the same way as described in the group C45 up to root canal filling with the calcium hydroxide and propylene glycol paste. Then, the palatal root surfaces and the alveolar wounds were treated with the lasers in the same way as described in the group L4, and the teeth were replanted into their respective sockets.

The GaAlAs laser device used in this study (BioWave LLLT; Kondortech Equipamentos Odontológicos Ltda., São Carlos, SP, Brazil) has the following characteristics: wavelength range from 660 nm (visible) to 830 nm (infrared), output power range from 30 mW (660 nm) to 40 mW (830 nm), CW or modulate CW (up to 1 kHz) operating modes, random beam polarization, 3 mm laser beam diameter on the handpiece tip, 0.07 cm^2 beam area, 20-degree beam divergence, and 1.5 m handpiece cable length.

After replantation, all animals received a single intramuscular 24 000 IU antibiotic dose (benzathine

benzylpenicillin – 12 000 IU, procaine benzylpenicillin – 6000 IU, potassium benzylpenicillin – 6000 IU, dihydrostreptomycin sulfate – 5 mg, streptomycin sulfate – 5 mg; Fort Dodge; Animal Health Ltda., Campinas, SP, Brazil).

The rats were sacrificed by anesthetic overdose 60 days after replantation. The anatomic pieces containing the replanted teeth were removed, fixed in 10% formalin for 24 h, decalcified in a 20% EDTA solution, pH 7.0, dehydrated, clarified and embedded in paraffin. Semi-serial longitudinal 6-μm-thick sections were obtained; part was stained with hematoxylin and eosin for histologic and histomorphometrical analyses under optical microscopy, and part was prepared for immunohistochemical analysis.

Histomorphometrical analysis

Images of the longitudinal sections were captured with a digital camera (Axio Cam MRc5; Carl Zeiss do Brasil Ltda., Rio de Janeiro, RJ, Brazil) attached to a stereomicroscope (Stemi 2000-C.; Carl Zeiss do Brasil Ltda.) with 1:20 increase, which provided a panoramic view of the tooth. The images were saved as figures in the Axio Vision 4.5 software (Carl Zeiss do Brasil Ltda.). ImageLab® 2000 image-analysis software (Diracom Bio Informática, Vargem Grande do Sul, SP, Brazil) was used for measurement of the resorption areas and ankylosis perimeter. For the histomorphometrical analysis, 10 sections were analyzed from 10 different slides of each experimental group. The mean percentage of resorption of the palatal dentin area was calculated according to each type of resorption, and data were entered into an Excel worksheet (Microsoft Corp., Redmond, WA, USA). For analysis of the areas of ankylosis, the perimeter corresponding to this type of repair was determined in relation to the total perimeter of the palatal surface, and the percent data were entered into an Excel worksheet as well for analysis. Scores from 1 to 4 were attributed to inflammatory resorption, replacement resorption and ankylosis, one being the best result, four being the worst result, and two and three occupying intermediate positions. Root resorption area: the areas of inflammatory and replacement root resorption were measured in representative slides. A four-point scoring system was used (6, 9), as follows: 1 – no resorption; 2 – 0.1 to 50% of the area with resorption; 3 – 51 to 99% of the area with resorption; 4 – 100% of the area with resorption. The perimeter of the ankylosed areas was measured in representative slides. A four-point scoring system was used (6), as follows: 1 – absence of ankylosis; 2 – 0.1 to 50% of the perimeter with ankylosis; 3 – 51 to 99% of the perimeter with ankylosis; 4 – 100% of the perimeter with ankylosis.

Immunohistochemical analysis

For the immunohistochemical analysis, four slides of each strain were selected for the immunohistochemical analysis, the expression of OPG, RANK, RANKL, and TRAP proteins was evaluated by the immunoperoxidase detection method (17, 18) (Figs 1c–f, 2c–f, 3c–f, 4c–f, 5c–f and 6c–f). A negative control was prepared for each specimen using the same method except for the primary antibody. A section representative of each protein in each

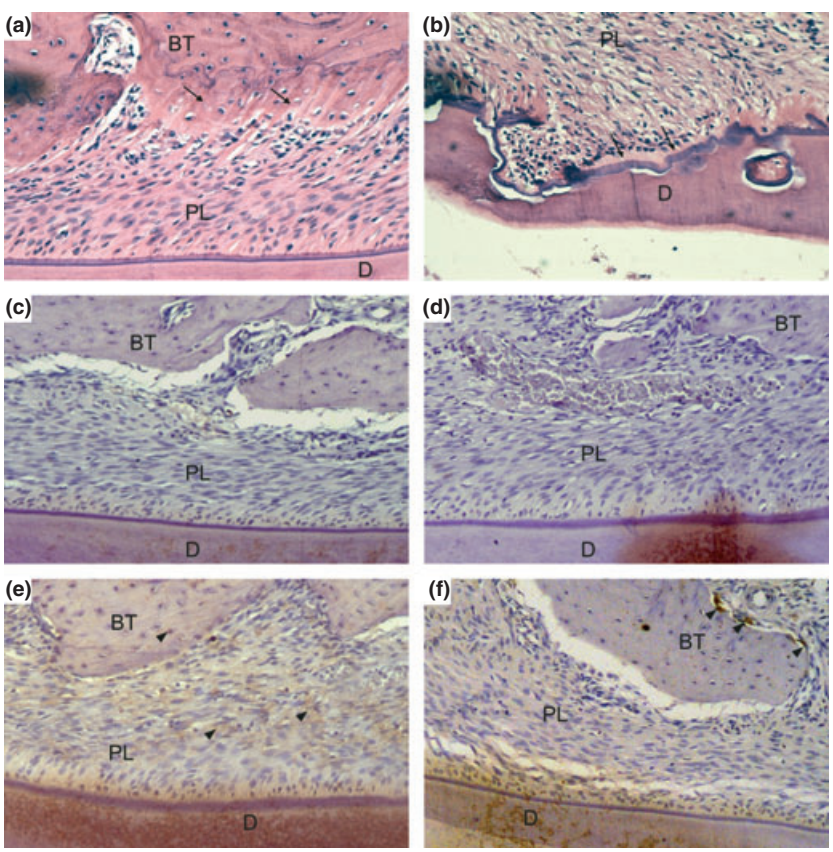

Fig. 1. Group C4 (4-min extra-alveolar time – control – immediate replantation) – Photomicrograph showing (a) alveolar bone wall with discrete new bone formation (arrows), periodontal ligament space filled by connective tissue with collagen fibers arranged exhibiting an oblique arrangement to root surface; (b) Resorption areas repaired by newly formed cementum (arrows) (H.E., original magnification 160×). Osteoprotegerin (c), RANK (d), RANKL (e), and TRAP (f) immunohistochemical expression (Original magnification 100×). Positive immunostaining (arrowheads). D, dentin; PL, periodontal ligament; BT, bone tissue.

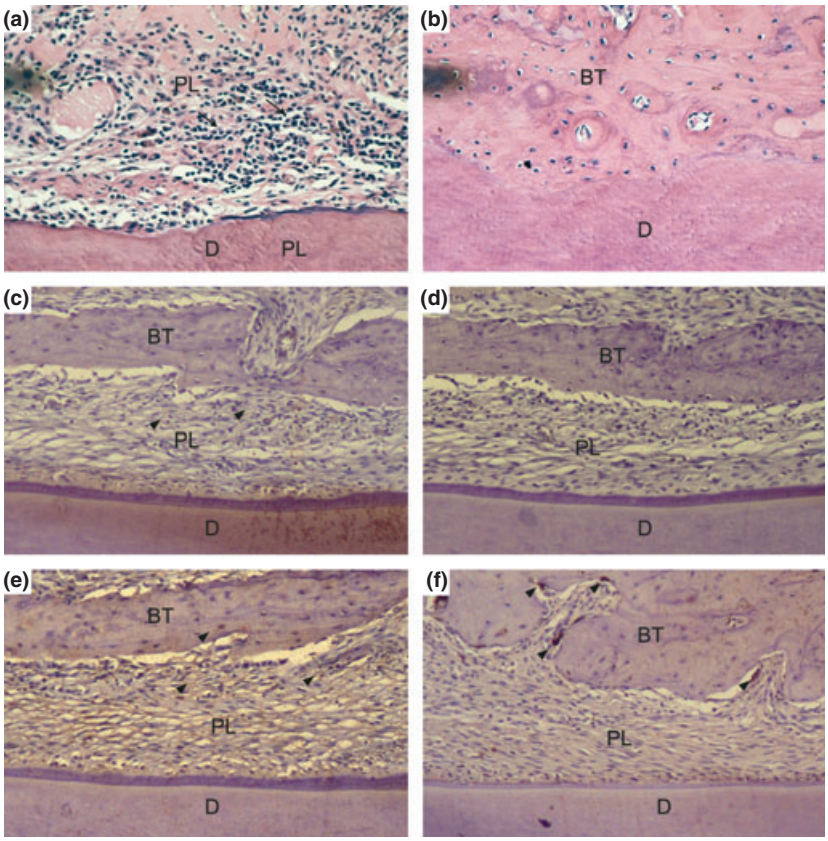

Fig. 2. Group L4 (4-min extra-alveolar time – laser – immediate replantation) – Photomicrograph showing (a) periodontal ligament (LP) exhibiting several inflammatory cells (arrows); (b) areas of replacement root resorption (H.E., original 160×). Osteoprotegerin (c), RANK (d), RANKL (e), and TRAP (f) immunohistochemical expression (Original magnification 100×). Positive immunostaining (arrowheads). D, dentin; PL, periodontal ligament; BT, bone tissue.

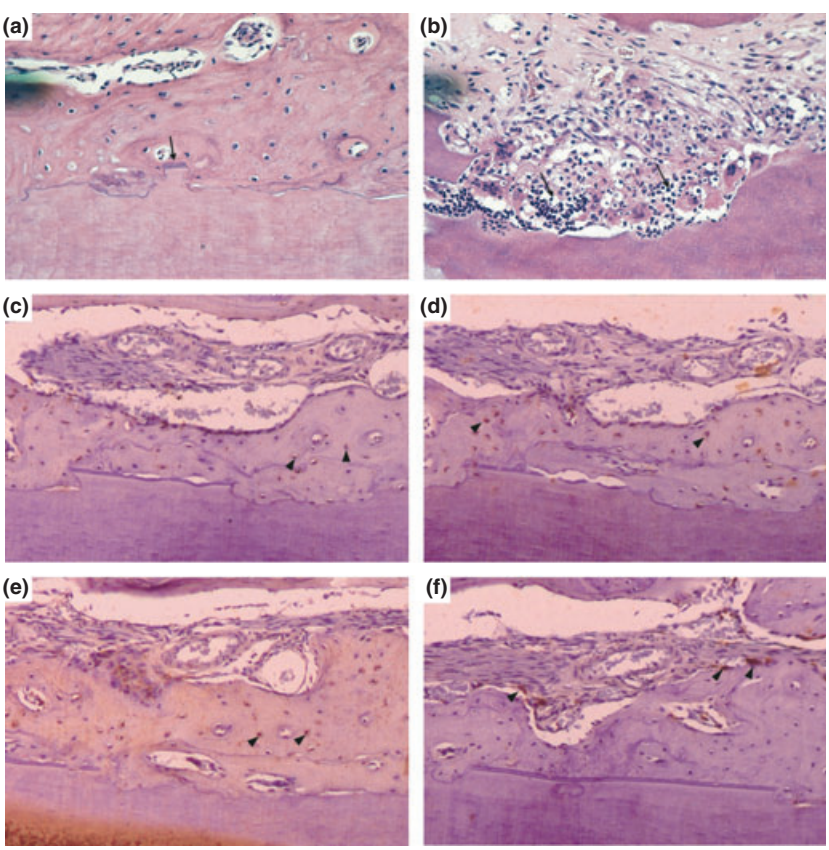

Fig. 3. Group C30 (30-min extra-alveolar time – control – delayed replantation) – Photomicrograph showing (a) replacement root resorption. The arrow indicates an area of ankylosis (arrow); (b) areas of inflammatory root resorption with numerous inflammatory cells (arrows) (H.E., original magnification 160×). Osteoprotegerin (c), RANK (d), RANKL (e), and TRAP (f) immunohistochemical expression (Original magnification 100×). Positive immunostaining (arrowheads). D, dentin; BT, bone tissue.

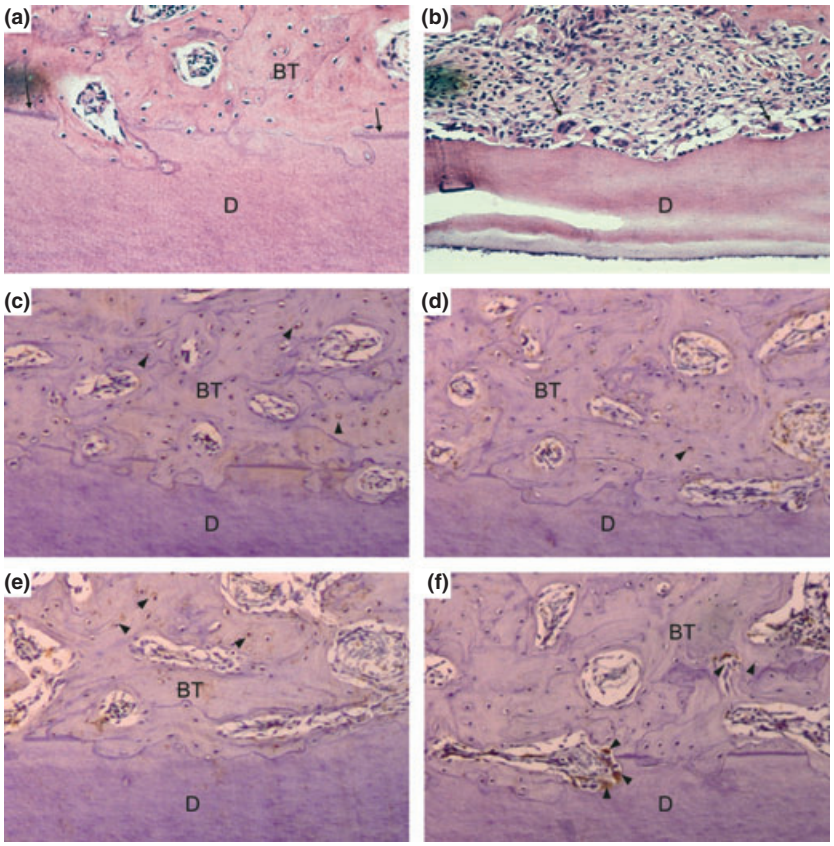

Fig. 4. Group L30 (30-min extra-alveolar time – laser – delayed replantation) – Photomicrograph showing (a) periodontal ligament space filled by bone tissue (BT). In some points, the cementum (arrows) is juxtaposed to the bone tissue (BT), and in other points the cementum layer suffered replacement resorption together with dentin (D); (b) dentin areas exhibiting inflammatory resorption with multinucleated cells (arrows) (H.E., original magnification 160×). Osteoprotegerin (c), RANK (d), RANKL (e), and TRAP (f) immunohistochemical expression (Original magnification 100×). Positive immunostaining (arrowheads).

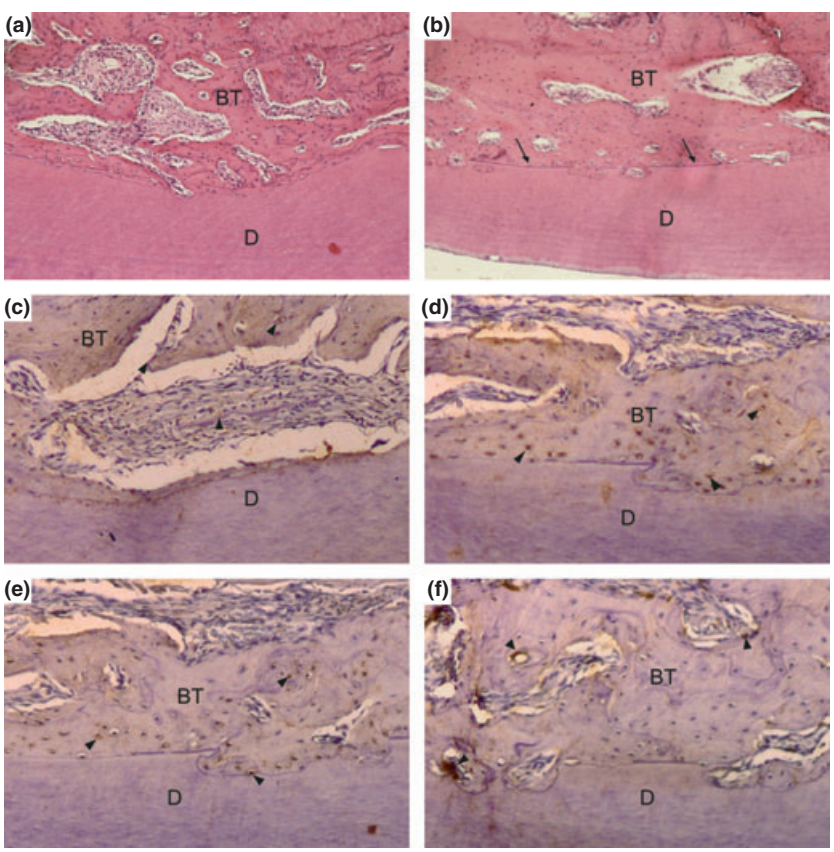

Fig. 5. Group C45 (45-min extra-alveolar time – control – delayed replantation) – Photomicrograph showing: (a) extensive areas of resorbed cementum and dentin (D) being replaced by bone tissue (BT); (b) area in which the periodontal ligament space is filled by bone tissue (BT). Dentin (D); cementum (arrows) (H.E., original magnification 160×). Osteoprotegerin (c), RANK (d), RANKL (e) and TRAP (f) immunohistochemical expression (Original magnification 100×). Positive immunostaining (arrowheads).

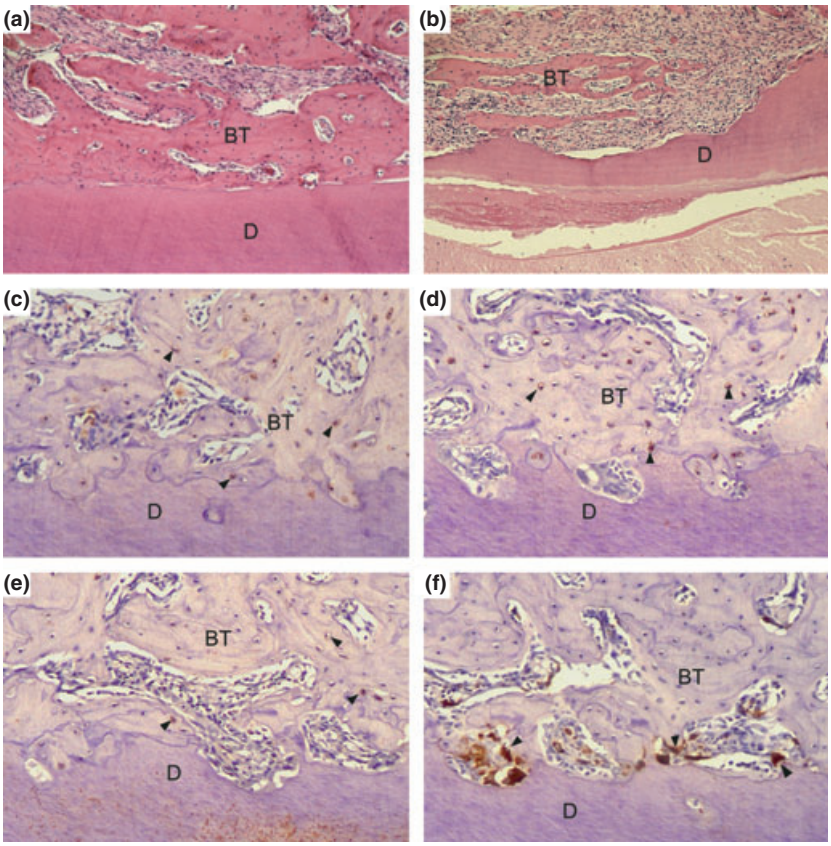

Fig. 6. Group L45 (45-min extra-alveolar time – laser – delayed replantation) – Photomicrograph showing (a) areas in which the periodontal ligament space is filled by bone tissue. Bone tissue (BT). Dentin (D); (b) areas of cementum and dentin resorption on root surface, with new formation of bone tissue (BT) (H.E., original 160×). Osteoprotegerin (c), RANK (d), RANKL (e), and TRAP (f) immunohistochemical expression (Original magnification 100×). Positive immunostaining (arrowheads).

specimen was captured by a digital camera (Axio Cam MRc5; Carl Zeiss do Brasil Ltda.) coupled to a stereomicroscope (Stemi 2000-C; Carl Zeiss do Brasil Ltda.) with 1:100 increase for analysis. The area corresponding to the palatal root dentin, PDL and palatal alveolar bone was examined.

The immunostaining intensity was classified by a calibrated examiner according to the following semi-quantitative scale (15): (−) absent or negligible staining, (+) weak staining, (++) moderate staining, (+++) strong staining. In order to facilitate intergroup comparisons, scores of 1, 2, 3, and 4 were attributed to absent/negligible, weak, moderate, and strong staining, respectively. The examiner was blinded to which groups the images belonged in order to avoid bias during the analysis.

Statistical analysis

In the histomorphometrical analysis, the scores corresponding to the percentages of inflammatory and replacement resorption and ankylosis obtained in the control and laser-treated groups were analyzed by the Mann–Whitney nonparametric test. In the immunohistochemical analysis, the obtained scores were analyzed by the Mann–Whitney and Kruskal–Wallis nonparametric tests. A significance level of 5% was set for all analysis. The control and laser-treated groups were compared for the immunohistochemical expression of RANK, RANKL, OPG and TRAP proteins at each extra-alveolar time. In addition, the balance between the expressions of the components of the RANK/RANKL/OPG system was analyzed in each group.

Results

During the course of the study, seven animals was lost (group C45: two, L4: two, L30: two, L45: one), which reduced the total sample size to 53, and the number of specimens in each group (C4 = 10, C30 = 10, C45 = 9, L4 = 8, L30 = 8, L45 = 9). In spite of these losses, the number of specimens *per* group ranged from 8 to 10, which is consistent with the number of specimens used in other studies investigating dental replantation (5–10).

The results were described after qualitative analysis under light microscopy of the following structures 60 days after tooth replantation: gingival mucosa, PDL, cementum, dentin, and alveolar bone wall. In all groups, epithelial reattachment occurred at the cemento-enamel junction level and the subjacent connective tissue showed few inflammatory cells.

C4 Group (4-min extra-alveolar time – laser). In most specimens, oblique PDL fibers were seen attaching to root surface, and no inflammatory cells were observed (Fig. 1a). In some regions of the root surface, cementum and dentin were resorbed and repaired by newly formed cementum (Fig. 1b). The discrete new bone formation on the alveolar bone wall did not cause narrowing of the PDL space.

L4 Group (4-min extra-alveolar time – laser). In some specimens, the PDL had several inflammatory cells (Fig. 2a). Cementum and dentin showed areas of

replacement (Fig. 2b) and inflammatory root resorption. The alveolar wall showed bone apposition causing narrowing of the PDL space.

C30 Group (30-min extra-alveolar time – control). In some areas, the PDL space was completely filled by bone tissue (Fig. 3a), while in others the connective tissue exhibited fibers arranged parallel to the root surface. Cementum and dentin presented areas of inflammatory (Fig. 3b) and replacement resorption. Few areas of ankylosis were observed (Fig. 3a). The alveolar wall showed deposition of newly formed bone, causing either narrowing or complete filling of the PDL space (Fig. 3a).

L30 Group (30-min extra-alveolar time – laser). In most specimens, the PDL space was filled by bone tissue along the three root thirds, characterizing the occurrence of new bone formation on the alveolar wall (Fig. 4a). The cementum was juxtaposed to the bone tissue in several areas along the root surface (Fig. 4a). The dentin exhibited areas of surface, inflammatory (Fig. 4b), and replacement (Fig. 4a) resorption.

C45 Group (45-min extra-alveolar – control). Great part of the PDL space was filled by bone tissue (Fig. 5a). Extensive areas of ankylosis were observed along the three root thirds (Fig. 5b). Cementum and dentin were either resorbed (with the presence of inflammatory cells) or replaced by bone tissue (Fig. 5a). The alveolar wall showed deposition of newly formed bone, causing either narrowing or complete filling of the PDL space.

L45 Group (45-min extra-alveolar – laser). PDL space was filled by bone tissue in almost the whole extension of the root (Fig. 6a). The PDL fibers were arranged parallel to the root surface. Extensive areas of the root surface exhibited cementum and dentin resorption (Fig. 6b). The alveolar wall showed bone apposition causing narrowing of the PDL space.

In the histomorphometrical analysis, the events of inflammatory resorption, replacement resorption and ankylosis were analyzed by comparing the control and laser-treated groups at each extra-alveolar time (Table 1). No statistically significant differences ($P > 0.05$) were found for inflammatory and replacement resorption (Table 1), but the presence of areas of ankylosis was significantly greater ($P < 0.05$) in L30 (Table 1).

Table 1. Mean scores and standard deviation (SD) for the histologic events in each group and significance (*P* value) after Mann–Whitney test for individual comparisons between the control and laser-treated groups at each extra-alveolar time

Histologic events	Extra-alveolar time (min)	Mean scores (SD) Control	Laser	*P* value
Ankylosis	4	1.10 (0.32)	1.37 (0.52)	0.1958
	30	1.60 (0.52)	2.12 (0.35)	0.0359*
	45	2.00 (0.53)	1.89 (0.33)	0.6638
Replacement resorption	4	1.30 (0.48)	1.62 (0.52)	0.1976
	30	1.90 (0.32)	1.87 (0.35)	0.9654
	45	1.87 (0.35)	2.11 (0.33)	0.1976
Inflammatory resorption	4	1.40 (0.52)	1.62 (0.52)	0.3839
	30	1.40 (0.52)	1.50 (0.53)	0.7618
	45	1.75 (0.46)	1.44 (0.53)	0.2367

*Statistically significant at 5%.

Table 2. Mean scores and standard deviation (SD) for protein immunostaining in each group and significance (*P* value) after Mann–Whitney test for individual comparisons between the control and laser-treated groups at each extra-alveolar time

Proteins	Extra-alveolar time (min)	Mean scores (SD) Control	Laser	*P* value
OPG	4	1.10 (0.32)	2.00 (0.75)	0.0066*
	30	2.90 (0.99)	3.00 (0.53)	0.7770
	45	2.87 (0.35)	2.67 (0.71)	0.3904
RANK	4	1.10 (0.32)	1.25 (0.46)	0.4497
	30	3.10 (0.32)	2.37 (0.52)	0.0059*
	45	3.50 (0.53)	2.89 (0.33)	0.0188*
RANKL	4	3.20 (0.79)	3.75 (0.46)	0.1219
	30	2.90 (0.32)	2.62 (0.74)	0.2374
	45	2.75 (0.71)	2.22 (0.44)	0.0967
TRAP	4	2.10 (0.32)	3.25 (0.46)	0.0003*
	30	1.90 (0.32)	2.50 (0.75)	0.0381*
	45	2.37 (0.52)	2.11 (0.33)	0.2393

OPG, osteoprotegerin; RANK, receptor activator of nuclear factor Kappa-B; RANKL, receptor activator of nuclear factor Kappa-B ligand; TRAP, tartrate-resistant acid phosphatase.
*Statistically significant at 5%.

In the immunohistochemical analysis, the scores attributed to the immunostaining for each protein (Table 2) were analyzed. Considering each protein alone at each extra-alveolar time, the comparison of control and laser-treated groups showed that the presence of positive immunostaining for OPG was significantly greater in L4 ($P < 0.05$, Table 2, Fig. 2c). Evidence of RANK immunostaining was greater in C30 and C45 ($P < 0.05$, Table 2, Figs 3f and 6f). Evidence of RANKL immunostaining was similar in the control and laser-treated groups at the three extra-alveolar times ($P > 0.05$, Table 2, Figs 1e, 2e, 3e, 4e, 5e and 6e). Greater evidence of TRAP immunostaining was observed in L4 and L30 ($P < 0.05$, Table 2, Figs 2f and 4f).

Analyzing OPG, RANK, and RANKL proteins at each extra-alveolar time, RANKL immunostaining predominated over RANK and OPG immunostaining in

Table 3. Mean scores and standard deviation (SD) for the proteins staining in each group and significance (*P* value) after Kruskal–Wallis test comparing the OPG/RANK/RANKL

Extra-alveolar time (min)	Group	Mean scores (SD) OPG	RANK	RANKL	*P* value
4	C4	1.10 (0.32)a	1.10 (0.32)a	3.20 (0.79)b	<0.001*
	L4	2.00 (0.76)a	1.20 (0.46)a	3.70 (0.46)b	0.0002*
30	C30	2.90 (0.99)a	3.10 (0.32)a	2.90 (0.32)a	0.6820
	L30	3.00 (0.53)a	2.37 (0.52)a	2.62 (0.74)a	0.1247
45	C45	2.88 (0.35)ab	3.50 (0.53)a	2.75 (0.71)b	0.0356*
	L45	2.67 (0.71)ab	2.89 (0.33)a	2.22 (0.44)b	0.0299*

OPG, osteoprotegerin; RANK, receptor activator of nuclear factor Kappa-B; RANKL, receptor activator of nuclear factor Kappa-B ligand.
*Statistically significant at 5%. Values with the same letter are not statistically different.

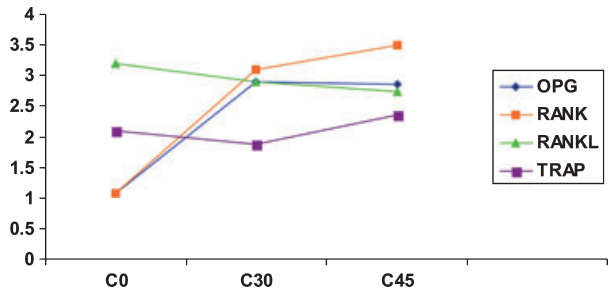

Fig. 7. Immunohistochemical expression of RANK, RANKL, osteoprotegerin, and TRAP proteins in the control groups (C4 – 4-min extra-alveolar time, C30 – 30-min extra-alveolar time, C45 – 45-min extra-alveolar time).

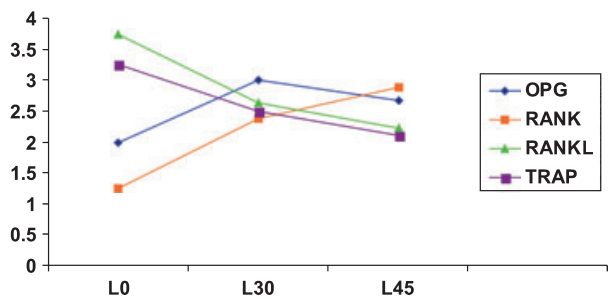

Fig. 8. Immunohistochemical expression of RANK, RANKL, osteoprotegerin, and TRAP proteins in the laser-treated groups (L4 – 4-min extra-alveolar time, L30 – 30-min extra-alveolar time, L45 – 45-min extra-alveolar time).

both groups with immediate tooth replantation ($P < 0.05$, Table 3, Figs 7 and 8). There was a balance in OPG, RANK, and RANKL immunostaining in the control and laser-treated groups with 30-min extra-alveolar time ($P > 0.05$, Table 3, Figs 7 and 8). For the 45-min extra-alveolar time, however, there was greater evidence of RANK over RANKL immunostaining for both control and laser-treated groups ($P < 0.05$, Table 3, Figs 7 and 8).

Discussion

In cases of dental trauma and replantation, areas of necrosis, blood clot, and inflammatory exudate are formed in the PDL fibers and PDL space. In the first hours, preosteoblastic cells migrate to these areas and differentiate into osteoblasts, which immediately start the secretion of bone matrix in a disorganized and accelerated manner. Deposition of this immature bone tissue occurs even on root surface areas that became unprotected after loss of precementum and cementoblasts because of the traumatic injury (19). Most studies on tooth replantation have used histologic and histomorphometrical methods to evaluate the healing process (6–9). The results of the present study confirm that immunohistochemistry is an important research tool that complements the histomorphometrical analysis by providing precise information on the expression and localization of each protein involved in the healing of replanted teeth. In addition, our findings confirm those of Manfrin (20) regarding the participation of the RANK-RANKL-OPG system in the healing process after tooth replantation.

Extra-alveolar times of 4, 30, and 45 min in saline storage were used in the present study to simulate immediate and delayed replantation based on the biostimulatory properties of LLLT, as it has been demonstrated that cemental PDL cells remain viable within these periods (19, 21). As the extra-alveolar time increased, it was possible to evaluate the effect of the LLLT on different amounts of viable PDL cells.

This study investigated the biostimulatory effect of GaAlAs diode laser, which can penetrate deep into tissues, and is portable, easy to use, and relatively inexpensive (11). Despite the lack of studies with a similar methodology, the laser parameter settings were selected taking into consideration the characteristics of the laser device regarding the active medium, handpiece cable length, and laser beam polarization, diameter, area, and divergence. The choice for the wavelength was based on the target tissue – PDL fibers. For the root surface-adhered PDL rests, visible light (630 nm) was used because it has less penetration potential and is more indicated for superficial lesions, while infrared light (830 nm) was used on the PDL rests adhered to the alveolar wound because it has greater penetration potential and may reach deeper tissues (22, 23).

Although the absorption of lasers emitted at the visible or infrared spectrum is mediated by different mechanisms – mitochondrial respiratory chain (24) and cellular membrane permeability (25), respectively, they have a similar interaction with the target biologic tissues. The biostimulatory effects of these lasers increase enzymatic activity, electron transport, and adenosine triphosphate (ATP) synthesis, which are favorable conditions for the occurrence of several cell metabolic reactions during the healing process (25, 26). However, in the present study, there was no evidence that the potential actions of LLLT on tissue healing, namely stimulation of cellular (27) and vascular (28) proliferation, antiinflammatory (24) and analgesic (28) effects, and biostimulation of bone remodeling (29–31), were beneficial to the repair after immediate and delayed tooth replantation.

The histomorphometrical analysis did not show significant differences between the control and laser-treated groups for the occurrence of inflammatory and replacement resorption, regardless of the extra-alveolar time. Accordingly, the immunohistochemical analysis showed equivalence for RANK and OPG immunostaining in the control and laser-treated groups at all extra-alveolar times. These results confirm the hypothesis that RANK is also signalized in bone formation events in models with an ongoing bone repair. Figures 7 and 8 show TRAP and RANKL lines with a similar behavior. This is a very positive finding because it demonstrates that RANKL signalizes and TRAP confirms the resorption.

The predominance of ankylosis in the group treated with laser and replanted after 30 min is likely due to an earlier deposition of bone tissue in this group. Supporting this assumption, the immunohistochemical analysis showed that the control group had very close scores for OPG and RANK. In the laser-treated group, although

no statistical significance was observed, there was a tendency to OPG immunostaining compared to RANK, which demonstrates a more advanced phase of the healing process. The predominance of RANK immunostaining in the control group and TRAP immunostaining in the laser-treated group is consistent with this statement. Previous reports affirm that direct application of LLLT favors the deposition of new bone on the alveolar walls (32–34). Although the main goal of this procedure was to stimulate the PDL cells remaining on the alveolar wound, the obtained results indicate a greater stimulation of bone cells, probably due to the small number of PDL cells present at the moment of photobiostimulation.

It is important to emphasize that the irregular formation of primary or immature bone on denuded root surfaces inside the alveolar wound of replanted teeth characterizes a primary event of ankylosis that might either be transitory or progress to a replacement resorption, depending on the viability of PDL cells (8).

In the present study, because of the recognized action of LLLT on osteoblast, fibroblast, macrophage, and lymphocyte proliferation and odontoblast differentiation and activation (26, 27), an accelerated cellular repair response was expected after irradiation of the root surface and the alveolar wound with a GaAlAs diode laser. In fact, the activation of osteoblasts would justify the earlier deposition of bone tissue, which was mainly observed in L30. The greater TRAP immunostaining in L4 and L30 confirmed the accelerated healing in these groups, as determined by its participation on cellular proliferation. The larger the number of viable cells, the more accentuated the differences observed for this protein.

On the other hand, this acceleration in the repair process in delayed replantation *per se* does not represent a benefit because the earlier the bone deposition in a denuded (PDL-free) surface, the earlier a replacement resorption process will initiate and consequently the faster the tooth will be lost.

It should be emphasized that the laser parameters, namely wavelength, output power, emission mode, application mode, number of applications, irradiated area, energy, irradiation time and energy density, have an important role in LLLT outcomes (35). Furthermore, it has been shown that its association with specific drugs or photosensitizers may also affect the results (36–38). Therefore, the effects of LLLT on dental traumatology need a more comprehensive investigation because of the large number of variables that may alter the response of the biologic tissues to laser irradiation. The findings of the present study must be interpreted as preliminary results.

In conclusion, under the tested conditions, the treatment of the root surface and the alveolar wound with a laser at low-level laser (GaAlAs diode laser) did not improve the healing process of immediate and delayed tooth replantation in rats. Further studies using different laser parameter settings are necessary to investigate any beneficial effects of LLLT on tooth replantation.

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