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Histological and immunohistochemical analyses of the chronology of healing process after immediate tooth replantation in incisor rat teeth

Sônia Regina Panzarini, Roberta Okamoto, Wilson Roberto Poi, Celso Koogi Sonoda, Denise Pedrini, Paula Ervolino da Silva, Celia Tomiko Matida Hamata Saito, Heloísa Fonseca Marão, Paulo Sedlacek

Department of Surgery and Integrated Clinic, Araçatuba Dental School, UNESP – Univ Estadual Paulista, Araçatuba, SP, Brazil

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Correspondence to: Sônia Regina Panzarini, Faculdade de Odontologia de Araçatuba, UNESP, Disciplina de Clínica Integrada, Rua José Bonifácio, 1193, Bairro Vila Mendonça, 16015-050 Araçatuba, SP, Brasil.
Tel.: +55 18 3636 3240
Fax: +55 18 3636 3332
e-mail: panzarin@foa.unesp.br

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Abstract – Dental tissues have special characteristics, and its regenerative capacity is noteworthy. However, understanding the circumstances that lead to regeneration is challenging. In this study, the chronology of the healing process after immediate replantation of rat incisor teeth was examined by histological and immunohistochemical analyses within a 60-day period. Thirty-six male Wistar rats had their maxillary right incisors extracted and replanted after 15 min in saline storage. The rats were sacrificed immediately 3, 7, 15, 28, and 60 days after replantation. The histological analysis showed rupture of the periodontal ligament and formation of a blood clot, which started being replaced by a connective tissue after 3 days. At 7 days, the gingival mucosa epithelium was reinserted and areas of root resorption could be seen. At 15 days, the periodontal ligament was repaired. At 3 days, the pulp presented an absence of the odontoblast layer, which started being replaced by a connective tissue. This tissue suffered gradual calcification, filling the root canal at 28 and 60 days. The root ends were closed. The immunohistochemical analysis revealed greater expression of OP, OPG, and RANK proteins in the initial periods (0 and 3 days), while TRAP expression predominated at 28 and 60 days ($P < 0.05$). In conclusion, in delayed tooth replantation, there is great new bone formation activity in the earlier periods of the repair process, while a predominance of bone resorption and remodeling is observed in the more advanced periods.

When a tissue is lacerated owing to a trauma, a sequence of events is unchained to repair the injured tissue. Although the healing process is basically the same in any part of the body, it may vary in some aspects depending on the involved tissues. Traumatic injuries to the teeth are particular complex because of the multiplicity of tissues and structures that may be affected (1). The goal of the healing process after traumatic injuries is to restore the continuity between the margins and reestablish the function of the tissue. It may occur by regeneration, when the structure and function of the lacerated or lost tissue are completely restored, or by healing, when the integrity of the lacerated or lost tissue is recovered by a new tissue, which does not restore the structure and function (1).

Dental tissues have special characteristics, and their regenerative capacity is noteworthy. Dentin, cementum, bone, and gingiva usually regenerate after trauma, while the pulp and periodontal ligament (PDL) may either regenerate or be repaired by the formation of reparative tissue or bone tissue (1).

Immunohistochemistry is a methodological tool that can be used to investigate how the healing process occurs after tooth replantation and help elucidating aspects that so far have more often been investigated histomorphometrically (2).

The proteins belonging to the tumor necrosis factor (TNF) superfamily—osteoprotegerin (OPG) (3), receptor activator of nuclear factor Kappa-B (RANK) (4), and its ligand (RANKL) (5)—are intimately linked to the dynamics of mineralized tissue resorption and remodeling processes. The balance between the expressions of the components of the RANK/RANKL/OPG system provides important information about bone metabolism (6, 7) 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 (8, 9).

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Manfrin (8) evaluated the immunostaining of OPG, RANK, and RANKL proteins in immediate and delayed replantation of rat teeth after 10 and 60 days, having as control group rat teeth in which no procedure was performed. In the control group, only OPG and RANKL proteins expressed, while in the other groups, OPG, RANK, and RANKL expression varied according to the analyzed period and type of replantation (immediate or delayed).

Saito et al. (6) also evaluated the expression of OPG, RANK, RANKL, and TRAP proteins in immediate replantation with or without application of laser after 60 days and observed that RANKL and TRAP had a statistically significant stronger expression than OPG and RANK.

Considering the results of these investigations, the aim of this study was to evaluate the chronology of the healing process after immediate replantation of rat teeth by histological and immunohistochemical analyses at 0, 3, 7, 15, 28, and 60 days postreplantation.

Material and methods

The research protocol was approved by the Animal Research Ethics Committee of the Araçatuba Dental School, São Paulo State University, Brazil (Protocol number 17/05).

Thirty-six male Wistar rats (*Rattus norvegicus, albinus*) weighing 250–300 g were used. The animals were fed ground solid ration (Ração Ativada Produtor®, Anderson & Clayton S.A., Laboratório Abbott do Brasil, São Paulo, SP, Brazil) and water *ad libitum*, except for the preoperative 12 h.

The surgical procedures were performed under general anesthesia. The animals received an intramuscular injection of xylazine hydrochloride (Anasedan; AgriBrands do Brasil Ltda., Paulínia, SP, Brazil—0.7 ml *per* 100 g body weight) to attain muscular relaxation and were anesthetized with ketamine hydrochloride (Dopalen; AgriBrands do Brasil Ltda.—0.7 ml *per* 100 g body weight). Asepsis of the anterior maxilla was performed with 1% iodine polyvinylpyrrolidone (Riodeine; Indústria Farmacêutica Rioquímica Ltda., São José do Rio Preto, SP, Brazil) followed by non-traumatic extraction of the maxillary right incisor of all animals.

The teeth were stored in sterile saline for 15 min. Thereafter, asepsis of the anterior maxilla was performed, the sockets were gently irrigated with sterile saline, and the teeth were replanted. 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 immediately 3, 7, 15, 28, and 60 days after replantation (six animals/period). The anatomic pieces containing the replanted teeth were removed, fixed in 10% formalin for 24 h, decalcified in a 4.13% EDTA solution, pH 7.0, and embedded in paraffin. Semi-serial longitudinal 6-μm-thick sections were obtained and stained with hematoxylin and eosin (H&E) for routine histological examination under optical microscopy.

Sections adjacent to the H&E-stained sections were used for immunohistochemical staining to determine the expression of OPG, RANK, RANKL, and TRAP proteins in the periodontal tissues during the healing process after immediate replantation. After deparaffinization, the slides used for immunohistochemical analysis were washed in phosphate buffered saline, followed by blocking with 0.03% hydrogen peroxide (Merck, Darmstadt, Germany) and antigen recovery. Prior to primary antibody incubation, the endogen biotin was blocked with non-fat milk.

The primary antibodies used in this experiment were goat polyclonal anti-OP, anti-OPG, anti-RANK, anti-RANKL, and anti-TRAP antibodies (M-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The secondary antibody was biotynilated anti-goat antibody (Pierce Biotechnology Inc., Rockford, IL, USA). The reactions were amplified by the streptavidin-biotin immunoperoxidase (Dako Corp., Carpinteria, CA, USA) technique and developed using diaminobenzidine (Dako Corp.) as chromogen. At the end of the immunohistochemical reactions, the slides were counterstained with Harris's hematoxylin, the specimens were dehydrated, and coverslips were mounted using Permount® (Fisher Scientific, Fair Lawn, NJ, USA). Immunostaining was evaluated along the entire root extension, PDL, and alveolar bone under conventional optical microscope.

Three slides of each specimen were selected for analysis of OP, OPG, RANK, RANKL, and TRAP expression by the immunoperoxidase detection method. A negative control was prepared for each specimen using the same method except for the primary antibody. Three sections representative of each protein in each tooth were captured by a digital camera (Axio Cam MRc5; Carl Zeiss do Brasil Ltda., Rio de Janeiro, RJ, Brazil) coupled to a stereomicroscope (Stemi 2000-C; Carl Zeiss do Brasil Ltda.) with 1:100 magnification. The area corresponding to the palatal root dentin, PDL, and palatal alveolar bone was examined. The immunostaining intensity of each protein was classified by a calibrated examiner according to the following semi-quantitative scale (6): (−) absent or negligible staining, (+) weak staining, (++) moderate staining, and (+++) strong staining. 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 the groups the images belonged to in order to avoid bias during the analysis.

Statistical analysis

In the immunohistochemical analysis, the non-parametric Kruskal–Wallis and Dunn's tests ($\alpha = 0.05$) were used to analyze the scores attributed to the immunohistochemical expression of RANK, RANKL, OPG, and TRAP proteins at each postreplantation time as well as the results of each individual protein at the different periods after replantation (immediate, 0, 3, 7, 15, 28, and 60 days).

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Results

Histological analyze

Immediate

The histological examination showed rupture of the gingival mucosa epithelium insertion at the cervical third. Along the palatal surface of the incisor, PDL was disrupted in its intermediate portion, with presence of blood clot between the cemental and the alveolar PDL (Fig. 1a). The alveolar PDL was richly vascularized and exhibited a large number of fibroblasts and well-organized collagen fibers that were inserted to the alveolar bone. In the cemental PDL, fibroblasts and cementoblasts could be seen close to the tooth surface and the cement was preserved in its entire extension. In the apical region, a blood clot was seen in intimate contact with the pulp stroma (Fig. 2a). The pulp tissue exhibited organized cells with normal characteristics and several blood vessels containing red blood cells. An organized and continuous odontoblast layer was seen over the predentin.

3 days

The histological examination showed that the epithelium of the gingival mucosa was partially reinserted to the root surface and the subepithelial connective tissue was well organized and free of inflammation. The bone crest

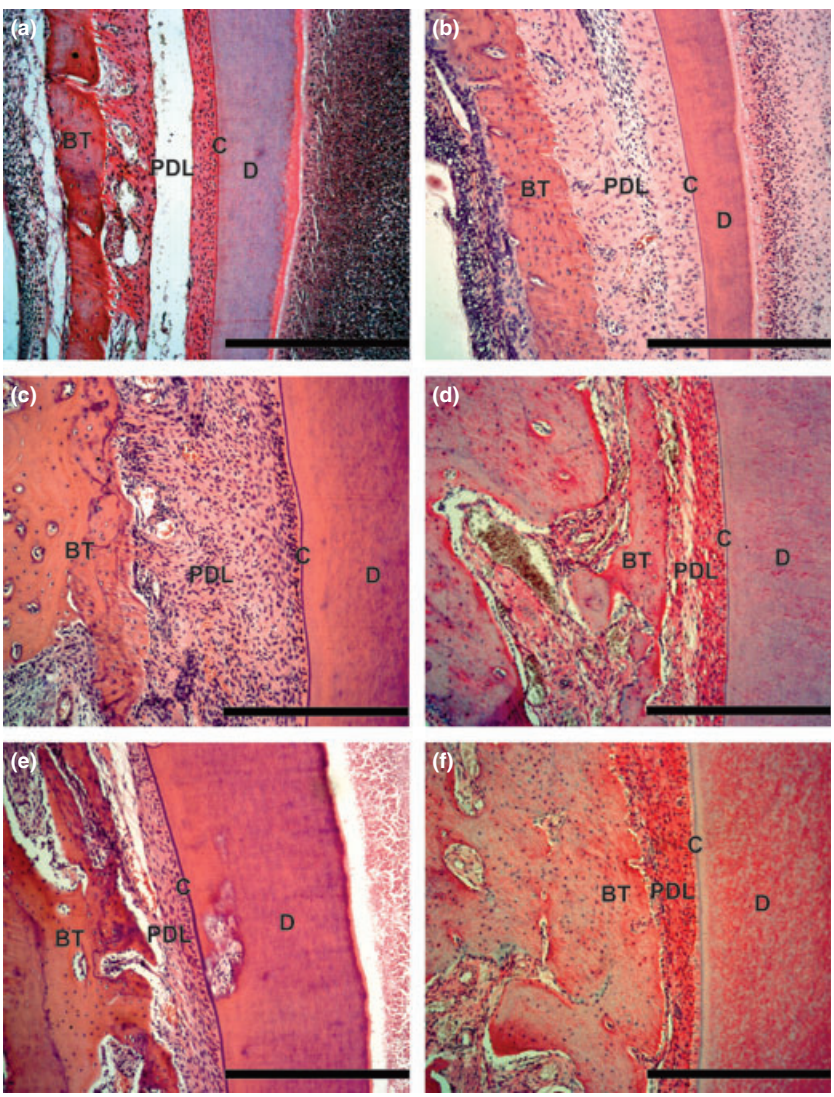

Fig. 1. Panel of photomicrographs illustrating the histological findings on the periodontal ligament (PDL) (Bar = 500 μm, original magnification 100×, HE). (a) 0 day – Rupture of the PDL in its middle portion, with presence of blood clot between the cemental and alveolar PDL. Dentin (D), Cement (C), Bone Tissue (BT). (b) 3 days – Middle portion of the PDL with collagen fibers disposed parallel to the root surface. Dentin (D), Cement (C), Bone Tissue (BT). (c) 7 days – PDL with collagen fibers inserted perpendicular to the cementum and alveolar bone. Dentin (D), Cement (C), Bone Tissue (BT). (d) 15 days – PDL fibers arranged perpendicular to the cementum and alveolar bone. Dentin (D), Cement (C), Bone Tissue (BT). (e) 28 days – Organized PDL. Dentin (D), Cement (C), Bone Tissue (BT). (f) 60 days – Presence of surface resorption. Dentin (D), Cement (C), Bone Tissue (BT).

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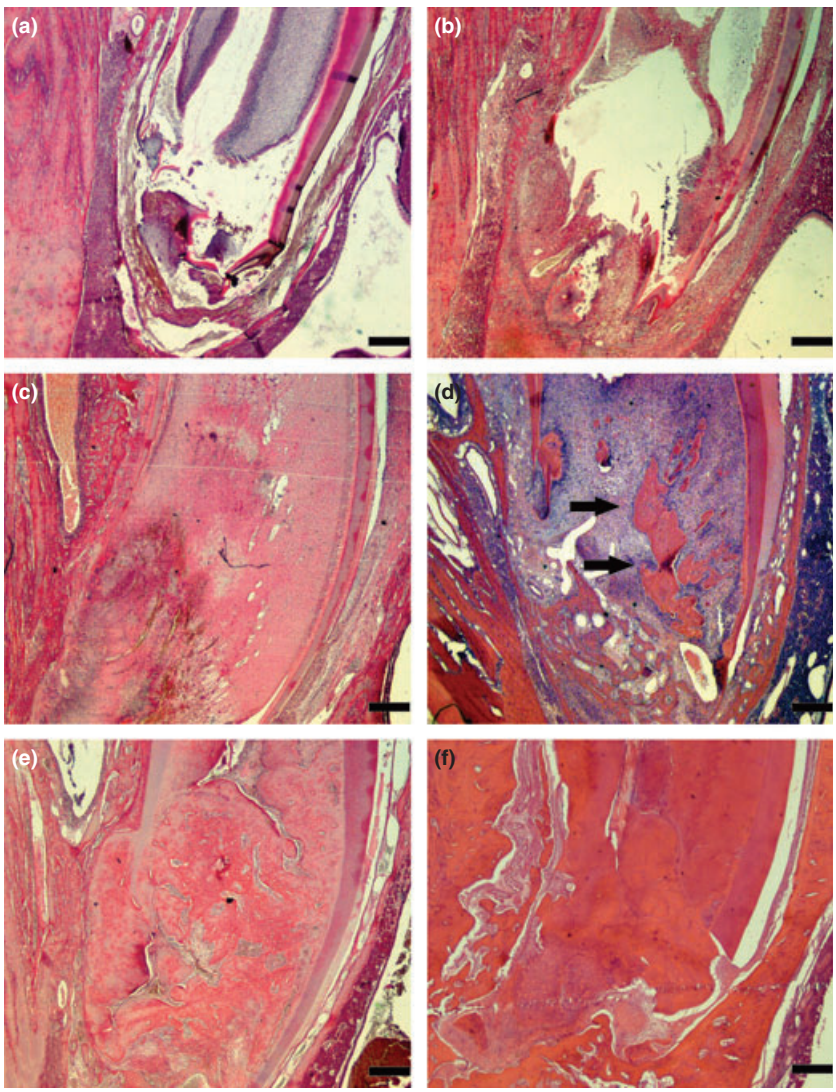

Fig. 2. Panel of photomicrographs illustrating the histological findings on the pulp tissue in the apical region. (Bar = 500 μm, original magnification 25×, HE). (a) 0 day – Presence of blood clot in intimate contact with the pulp stroma. 25×. (b) 3 days – Pulp tissue with normal characteristics and several blood vessels containing red blood cells. (c) 7 days – Part of the pulp stroma in the apical region replaced by a richly vascularized connective tissue. (d) 15 days – Apical pulp connective tissue being replaced by calcified tissue (arrow). (e) 28 days – Zones of calcification inside the root canal. (f) 60 days – Pulp stroma completely replaced by calcified tissue.

presented areas of active resorption. The alveolar PDL exhibited few cells with pycnotic nuclei and amorphous collagen fibers. Large osteoblast-like cells and undifferentiated mesenchymal cells could be seen in the entire extension of the alveolar PDL close to the blood vessels. The alveolar bone was organized and presented several young osteoblasts and small areas of active resorption. Few fibroblasts and cementoblasts were observed in the cervical third of the cemental PDL. There were few collagen fibers inserted to the cementum, with predominance of those that were disposed parallel to the root surface. There were a large number of fibroblasts, cementoblasts, and collagen fibers parallel to the root surface in the apical and middle thirds of the root (Fig. 1b). In the cervical third, the intermediate PDL layer exhibited a large fibrin mesh, a large number of red blood cells, macrophages, and discrete fibroblast proliferation. An amorphous pulp tissue with pycnotic cells and no odontoblast layer was observed. The predentin was preserved in the entire extension. In the apical third and part of the bottom of the socket, there was intense fibroblast activity, presence of macrophages, and replacement of part of the pulp stroma by a connective tissue rich in blood vessels and young fibroblasts (Fig. 2b).

7 days

The histological examination showed that the gingival mucosa epithelium was reinserted to the root surface and covered by parakeratin. The subepithelial connective tissue was free of inflammation close to the root surface and on the remodeled alveolar bone crest. There was an intense osteoblast activity along the entire extension of the alveolar bone. The alveolar PDL fibers were inserted

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perpendicular to the alveolar bone, which did not exhibit areas of active resorption. The cemental PDL presented a large number of fibroblasts. Cementoblasts were seen close to the cementum, which was preserved in most part of the root, and only small areas of surface resorption were observed. In the apical third, the intermediate portion of the PDL presented intense fibroblast proliferation and newly formed collagen fibers joining the cemental and alveolar PDL (Fig. 1c). In the middle third, there was connective tissue with well-differentiated collagen fibers and newly formed bone trabeculae. In the cervical third, there was a predominance of a loose connective tissue rich in blood vessels and fibroblasts. Most part of the pulp tissue was amorphous, with pycnotic cells and absence of the odontoblast layer. However, the predentin and the odontoblast layer were preserved in some areas. In the apical third, starting from the bottom of the socket, there was an intense vascular proliferation and loose connective tissue rich in fibroblasts replacing the pulp stroma (Fig. 2c).

15 days
The histological examination showed that the gingival mucosa epithelium presented similar characteristics to those found at 7 days. The PDL was reorganized, and the site of rupture could not be visualized in its middle portion, which exhibited collagen fibers parallel to the root surface. The cemental and alveolar PDL fibers were inserted perpendicular to the cementum and the alveolar bone, respectively (Fig. 1d). Cementum was preserved on the root surface except for small areas with active root resorption. There was an intense osteoblast activity along the entire extension of the alveolar bone. In the apical region of the root, there was intense new bone formation and a large number of bone trabeculae replacing the pulp connective tissue (Fig. 2d). In the cervical region of the socket, the amorphous pulp stroma presented pycnotic cells. New bone formation and dystrophic calcification in the pulp core were observed in the middle and apical regions of the root. Newly formed dentin tissue and several odontoblasts were seen close to the dentin surface.

28 days
The histological examination showed that the gingival mucosa epithelium had similar characteristics to those seen at 7 and 15 days. The PDL was reorganized and presented collagen fibers parallel to the root surface in its middle portion (Fig. 1e). Along the palatal root surface, there was intense osteoblast activity close to the alveolar bone and narrowing of the PDL space. A fibrous connective tissue was observed, and the blood vessels close to the alveolar bone had thick walls. The cementum was preserved in a great part of the root surface, but some areas of active root resorption could be seen. The pulp stroma up to the middle third of the root was replaced by newly formed connective tissue, with a large number of fibroblasts, osteoblasts, and bone trabeculae. Dystrophic calcifications were seen in the pulp core and periphery (Fig. 2e). Close to the dentin wall, there was a thick layer of newly formed dentin and predentin, just underneath the remaining odontoblasts.

60 days
The histological examination showed that the gingival mucosa epithelium had similar characteristics to those seen at 7 and 15 days. Fibroblasts were disposed parallel to the root surface in the intermediate portion of the PDL, which was repaired. Resorbed areas repaired by cementum were observed in some areas of the root surface, characterizing a surface resorption (Fig. 1f). In other areas, the root tissue was replaced by bone tissue. The PDL space was narrowed. In some specimens, the pulp stroma was completely replaced by calcified tissue in its entire extension (Fig. 2f).

Immunohistochemical analysis

Immediate
The immunohistochemical analysis revealed intense positive immunostaining for OP, OPG, RANK, and RANKL in the extracellular matrix of the PDL connective tissue and in the alveolar bone, as well as weak immunostaining for TRAP (Fig. 3a).

3 days
The immunohistochemical analysis revealed decrease in immunostaining for OP, RANK, and RANKL. Strong immunostaining for OPG was maintained, while evidence of immunostaining for TRAP remained weak (Fig. 3b).

7 days
The immunohistochemical analysis revealed positive immunostaining for PDL cells such as fibroblasts and cementoblasts. There was greater immunostaining for OP and RANK, weaker immunostaining for OPG and RANKL than at 3 days, and stronger immunostaining for TRAP near to the PDL compared with the earlier period (Fig. 3c).

15 days
The immunohistochemical analysis revealed strong positive immunostaining of the PDL cells for OP, OPG, and RANK and weaker immunostaining for RANKL compared with the earlier period. Evidence of immunostaining for TRAP remained strong (Fig. 3d).

28 days
The immunohistochemical analysis revealed weak immunostaining for OP, RANK, and RANKL and moderate for OPG and TRAP (Fig. 3e).

60 days
The immunohistochemical analysis revealed weak immunostaining for OP, moderate for OPG, RANK, and RANKL, and strong for TRAP (Fig. 3f).

Statistical analysis

Analyzing OP, OPG, RANK, and RANKL proteins at each postreplantation time, there was significantly greater ($P < 0.05$) positive immunostaining for OP at 0 day than at 60 days (Fig. 3). Immunostaining for OPG was significantly greater ($P < 0.05$) at 3 days than at

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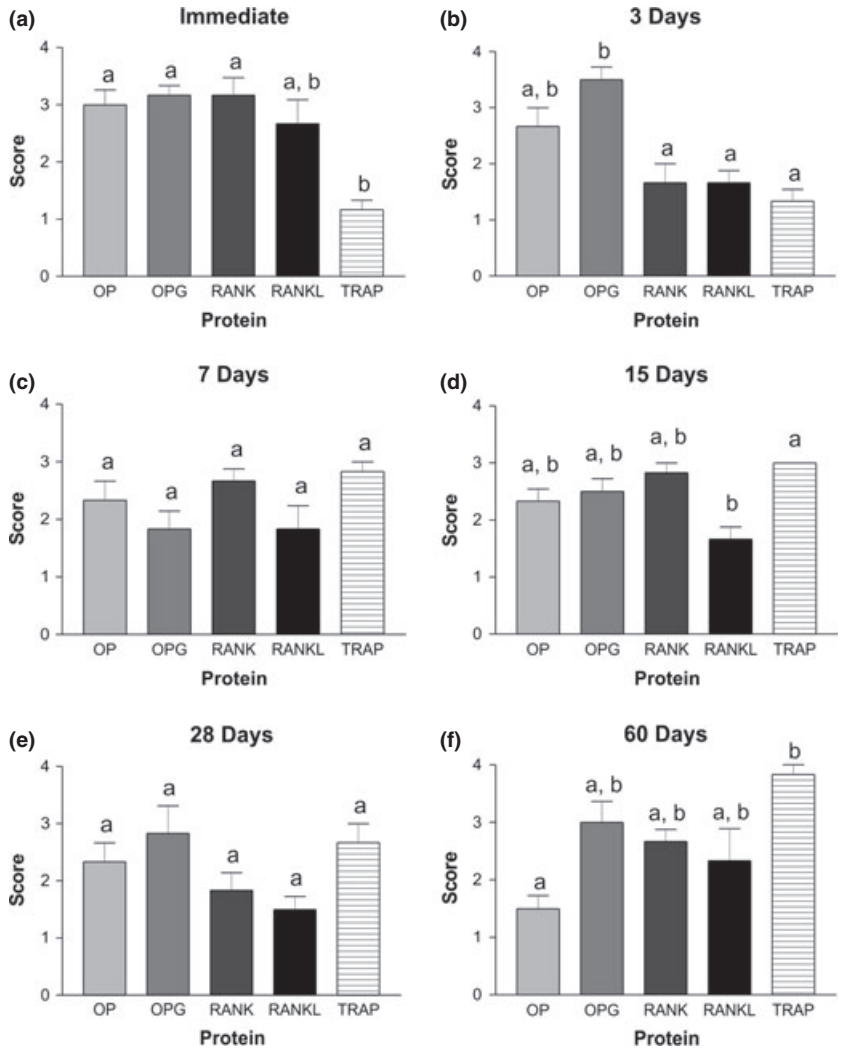

Fig. 3. Expression of the different proteins immediately (a), 3 days (b), 7 days (c), 15 days (d), 28 days (e), and 60 days (f) after replantation. Bars with the same letter do not differ significantly among each other (Kruskal–Wallis test; $P > 0.05$).

7 days (Fig. 3). Immunostaining for RANK was significantly greater ($P < 0.05$) immediately after replantation than after 3 days. There was statistically significant difference ($P < 0.05$) for TRAP immunostaining comparing the following postreplantation times: immediate vs 60 days and 3 days vs 60 days (Fig. 3). Positive immunostaining for RANKL was statistically similar ($P > 0.05$) for all extra-alveolar times.

OP, OPG, RANK, RANKL, and TRAP expression at the different postreplantation times can be seen in Fig. 4.

Discussion

Understanding the circumstances that lead to the regeneration of oral tissues has been a major challenge for dental research. It is known that a great variety of signals are released when an injury occurs, inducing neighboring cell populations to respond with proliferation, migration, or differentiation (1).

OP is a bone matrix protein that, in addition to binding strongly to hydroxyapatite crystals, have binding sites for the integrins present on the surface of osteoblasts and osteoclasts, thus promoting adhesion of these cells to the bone (10). It is released from the bone during demineralization (11), which is a probable explanation for its high expression at 3 days after replantation, when the histological examination showed areas of active resorption in the bone crest.

Current research has focused on elucidating the biomolecular mechanism of root resorption, especially by evaluating the expression of the RANK/RANKL/OPG system components. So far, it has been demonstrated by immunohistochemical analyses that the RANK receptor is expressed by odontoblasts, while the RANKL receptor is expressed by pulp odontoblasts, PDL fibroblasts, and cementoblasts (12–14). Fukushima et al. (15) have found that, during the physiological root resorption, PDL cells express RANKL but decrease OPG expression. Expression of RANKL is likely to participate in odontoclastogenesis and activation of physiological root resorption. This way, the intensity of the clastic activity seems to be regulated by systemic

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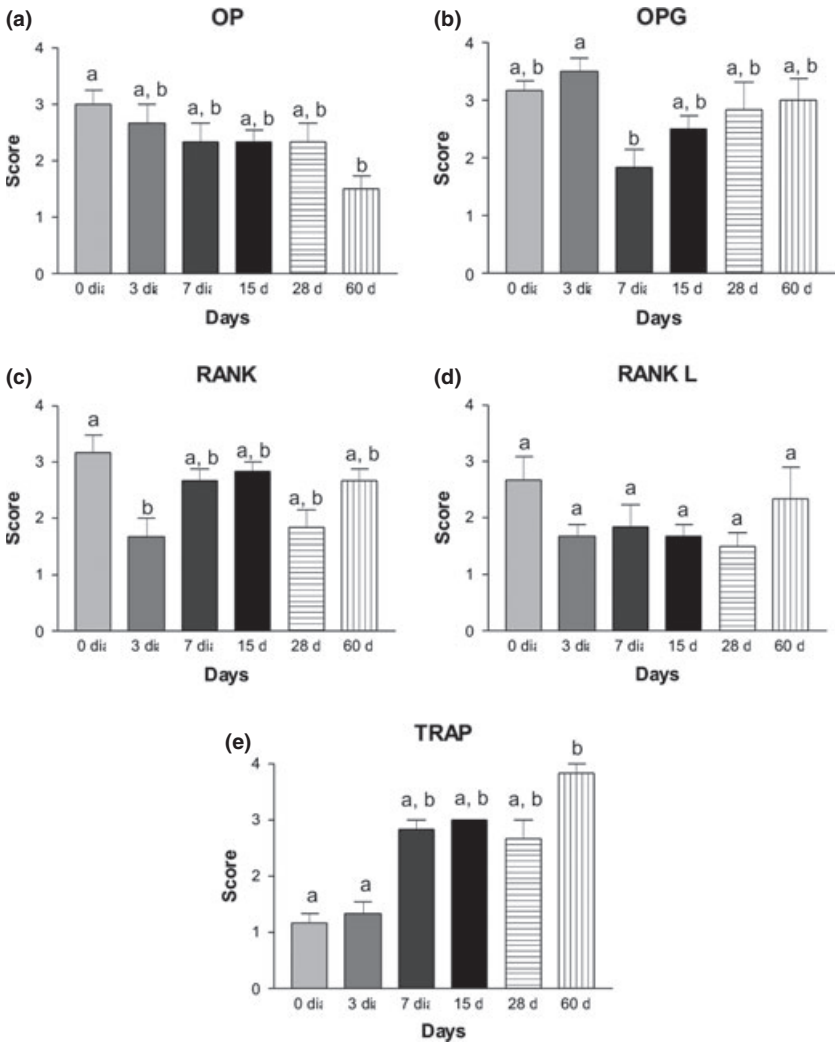

Fig. 4. OP (a), OPG (B), RANK (c), RANKL (d), and Tartrate-resistant acid phosphatase (e) expression at the different postreplantation times. Bars with the same letter do not differ significantly among each other (Kruskal–Wallis test; $P > 0.05$).

(endocrines) and local (cytokines) factors mediated by the RANK/RANKL/OPG system. In this study, there was greater RANKL expression at 60 days postreplantation. This period had greater clastic activity, as demonstrated by the strong TRAP expression.

Manfrin (8) found values for OPG, RANK, and RANKL expression in the immediate replantation group at 10-day postreplantation time similar to those obtained in this study at 15-day postreplantation time. However, OPG and RANK expression was significantly higher immediately and 3 days after replantation. This result confirms by immunohistochemistry the migration and differentiation of odontoblastic cells in the first hours after replantation, which deposit bone matrix typical of immature bone on root surface. However, the decrease in local factors such as inflammatory cytokines because of PDL regeneration and pulp revascularization reduced the expression of these proteins at 28 and 60 days. These results are similar to those of a recent study that evaluated immunohistochemically PDL cells after immediate replantation of rat teeth (2, 6, 8). The authors found that both cell proliferation and apoptosis occur in different patterns and at different times to maintain regular spacing of the PDL after tooth replantation (16).

Osteoclasts are readily distinguished from macrophages by the presence of TRAP in their cytoplasm (19). This type-V isoenzyme of acid phosphatase (17) presents an intense activity in osteoclasts, being considered a specific marker for osteoclasts (18, 19). In this study, there was a significant increase in TRAP expression from day 7 to the later periods, reaching the peak at 60 days, at which time areas of active root resorption were observed histologically, confirming the presence of clastic cells.

This study on the chronology of the repair process in immediate tooth replantation revealed that PDL regeneration may occur, leading to preservation of the teeth and absence of external root resorption. There was no statistically significant difference in protein expression between 28 and 60 days, which indicates that PDL repair is complete at 28 days and that there are no remarkable changes at 60 days.

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Another factor that contributed to the low incidence of root resorption was the occurrence of pulp revascularization, which was probably due to the short extra-alveolar time and the wide apical foramen of rat teeth, which favors the revascularization process. Pulp necrosis would start a pathological root resorption process by the persistence of local inflammatory agents (20, 21, 22).

The results of this 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.

Conclusion

The findings of the histological and immunohistochemical analyses confirmed that in cases of delayed tooth replantation, there is great new bone formation activity in the earlier periods of the repair process, while a predominance of bone resorption and remodeling is observed in the more advanced periods.

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