Effect of denervation on healing after tooth replantation in the ferret

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Studies have shown that the sensory nerves participate in inflammation and immune responses and possess trophic-facilitating wound healing in general. Tooth avulsion represents a pulpal and periodontal injury, and the mechanisms involved in the healing responses subsequent to replantation of teeth are still unclear. The objective of this study was to investigate the healing responses after denervation and replantation of teeth. Unilateral denervation was performed in 15 ferrets by axotomy of the inferior alveolar nerve, 5 days before extraction of the first lower premolars. Six weeks later the mandibles were excised and processed for histological evaluation. Immunohistochemistry was performed using antibodies against the sensory neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP), and measurements of root resorption and ankylosis were performed in four sections from each replanted tooth. After 6 weeks substantial reinnervation was observed in the jaws. Immunoreactivity in the pulp was observed in only two replanted teeth on the denervated side, compared with four on the innervated side. Total pulp necrosis appeared in 10 replanted teeth on the denervated side and in 5 on the innervated, indicating that sensory nerves promote survival of the pulp after replantation. SP-immunoreactive (IR) fibers were more frequently observed in the resorptive lacunae than CGRP-IR fibers. However, resorptive areas lacking IR fibers were frequently found along the root surface. Root resorption averaged 0.062 ± 0.029 mm^2 on the innervated side compared to 0.016 ± 0.0043 mm^2 on the denervated ($P < 0.02$). Ankylosis was observed in four of the replanted teeth on the innervated side (169.3 ± 49.7 μm) and in six on the denervated side (332.56 ± 193.2 μm) ($P = 1$). It is concluded that the sensory nerves promote root resorption after pulpoperiodontal injuries but have less influence on the osteoblastic activity expressed by ankylosis.

☐ *Ankylosis; immunohistochemistry; neuropeptide; root resorption*

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In recent years much attention has been given to the participation of sensory nerves in inflammatory and immunological responses. There is growing evidence that neuropeptides, such as substance P (SP) and calcitonin gene-related peptide (CGRP), which are released from peripheral sensory nerve endings, are involved in these responses by exerting several regulatory functions (1, 2). Their biological actions are wide and seem to include most parts of the inflammatory and healing responses after injury. These effector responses include vasodilatory and vascular permeability-increasing effects (1, 3), a chemotactic effect on neutrophils, macrophages, and T lymphocytes (4), improved wound healing potential (5), increased survival of critical flaps (6, 7), and stimulation of angiogenesis (8). In vitro studies have shown that SP and CGRP stimulate osteogenesis (9) and that CGRP inhibits bone resorption (10, 11). In contrast, SP has been found to enhance osteoclast-induced bone resorption activity in culture (12). Osteoclasts have been thought to derive from cells of the monocyte–macrophage lineage (13), and as alveolar macrophages they have been shown to possess neurokinin (NK) receptors (14) and also CGRP receptors (13, 15).

Tooth avulsion represents a pulpoperiodontal injury, and the role for sensory nerves in the healing responses subsequent to replantation of teeth are still presently unclear. As both ankylosis and root resorption are frequently observed after tooth replantation (16), the aims of this study were: 1) to observe the effect of sensory nerves on hard-tissue responses, and 2) to identify sensory nerve markers (CGRP and SP) and their localization in the injured tissues by immunohistochemistry.

Materials and methods

A total of 15 young ferrets (10 female and 5 male, 1–2.3 kg body weight) were anesthetized with 1 ml/kg body weight ketamine hydrochloride (Ketalar®, 1 mg/ml) mixed with 0.1 ml/kg b.w. medetomidine hydrochloride (50 mg/ml) administered intramuscularly. The animal experiment was approved by the local ethical committee and was in accordance with the recommendations given by the Norwegian State Commission for Laboratory Animals.

Unilateral surgical denervation of the inferior alveolar nerve (IAN) was performed under a microscope through an intraoral buccal incision behind the last molar on the left side of the mandible. The soft tissue and periosteum were retracted, and the mandibular bone exposed. The IAN was thereafter exposed by drilling a cavity with a round dental bur, ISO 310.204.001001.023 (Komet, Germany), in a low-speed handpiece, cooled with saline, and elevated with a curved probe. To delay reinnervation in the time course of the experiment, approximately 3 mm of the nerve was carefully removed, without bleeding from the blood vessels. The wound was closed with two to three

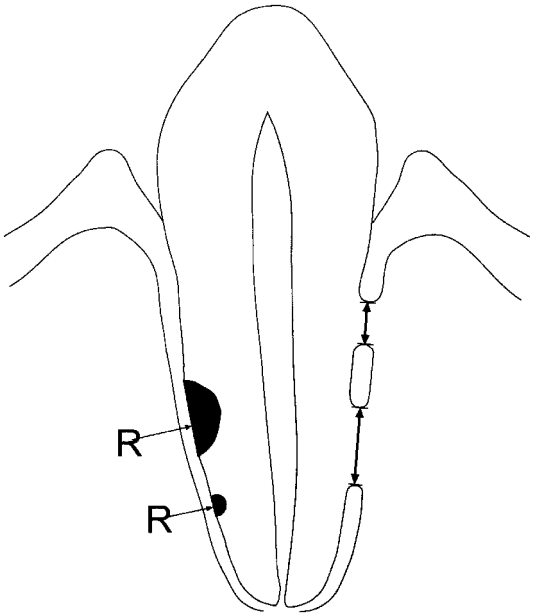


Fig. 1. Schematic drawing of the replanted first premolar indicating the areas for measurements. The total area of root resorption (R) was measured in square millimeters. Ankylosis was measured in micrometers as the total length of fused bone and root.

sutures in the vestibular mucosa. An antibiotic (streptocillin vet., 1 ml/kg b.w., Boehringer Ingelheim, Germany) and an analgesic (buprenophine (Temgesic®, Reckitt & Colman, UK), 0.15 ml/kg b. w.) were administered intramuscularly after denervation. The animals were left to recover for 4–5 days before they were reanesthetized. The first mandibular premolars were extracted bilaterally with an elevator and replaced into the sockets after less than 1 min. The ferrets received another equal dose of antibiotic and analgesic and were observed for 6 weeks. The animals were kept on soft diet for 24 h; thereafter they received a standard diet.

At the end of the observation period all animals were deeply anesthetized with an overdose of ketamine/medetomidine. Transcardiac perfusion was done with phosphate-buffered saline (PBS) containing 0.003% heparin followed by 4% paraformaldehyde and 0.2% picric acid in 0.1M phosphate buffer, pH 7.4. The mandibles were excised, postfixed for 24 h, and demineralized in 4N formic acid and 0.05 M sodium formate at 4°C, for 2–3 weeks.

Table 1. Pulp conditions in denervated and innervated replanted teeth ($n = 24$)

	Total necrosis	Partial necrosis +IR fibers	Partial necrosis −IR fibers	Vital pulp
Denervated	10	1	0	1
Innervated	5	3	2	2

Immunohistochemistry

After demineralization the specimens were rinsed in PBS for 24 h and saturated in 30% sucrose in 0.1 M phosphate buffer for another 24 h. The jaws were serially sectioned in a saggital plane at 40 µm on a freezing microtome. The 40-µm sections were free-floating in tissue culture wells and reacted for visualization of immunoreactive (IR) nerve fibers. Alternate serial sections from control and contralateral denervated jaws were incubated for 72 h with polyclonal antibody to rat CGRP (1:6000 dilution) or SP (1:5000 dilution) (Cambridge Research Biochemicals, Cambridge, UK). The antigen–antibody complex was localized by the avidin–biotin peroxidase reaction, using a commercially available ABC kit (Vectastain ABC kits, Vector Laboratories Inc., Burlingame, Calif., USA) and visualized by means of 3′3-diaminobenzidine (Sigma Chemical Co., St Louis, Mo., USA) in the presence of 0.2% $(NH_4)_2Ni(SO_4)6H_2O$) to enhance the chromogen staining.

After several rinses in PBS the sections were mounted on gelatin-coated slides, air-dried, and then counterstained with methylene blue/azure II in 1% sodium borate and distilled water. Thereafter they were dehydrated in graded alcohols, cleared in xylene, and coverslipped with Eukitt (O. Kindler, Freiburg, Germany). Immunocontrols were routinely performed by replacing the primary or secondary antibody with PBS.

Evaluation

Observations and photomicrography were done with

Fig. 2. Calcitonin gene-related peptide-immunoreactive fibers (arrows) in the pulp chamber (P) of a replanted premolar from the innervated side of the mandible. D = dentin. Bar = 50 µm.

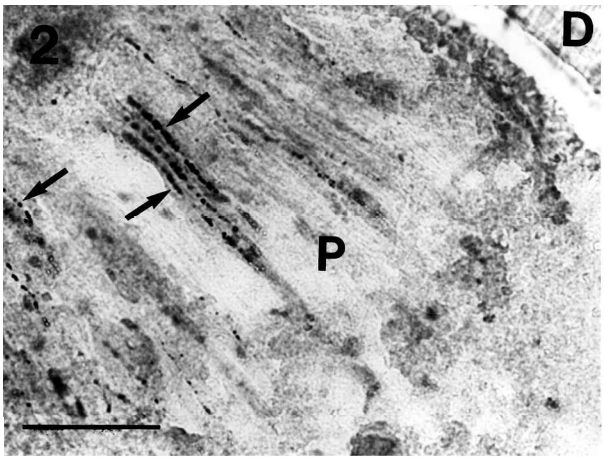
Fig. 3. Reinnervation of calcitonin gene-related peptide-immunoreactive fibers (arrows) in the pulp (P) of a replanted tooth from the axotomized side of the jaw. Mast cells (arrowheads) could frequently be observed close to reinnervating nerve fibers. Note the lack of odontoblastic layer. Bar = 50 µm.

Fig. 4. Calcitonin gene-related peptide-immunorecative fibers (arrows) in regenerated periodontal ligament (PDL) of a replanted first premolar. Section from an innervated tooth. B = bone; D = dentin. Bar = 50 µm.

Fig. 5 Immunoreactive fibers for calcitonin gene-related peptide (arrows) located in bone resorption adjacent to the periodontal ligament (PDL) of a replanted premolar on the innervated side of the jaw. B = bone. Bar = 50 µm.

Fig. 6. Substance P-immunoreactive nerve fiber (arrows) in area of root resorption. Section from the innervated side of the mandible. D = dentin. Bar = 50 µm.

Fig. 7. Resorption lacunae from the denervated side, incubated with antibody for calcitonin gene-related peptide. D = dentin; V = vessel. Bar = 50 µm.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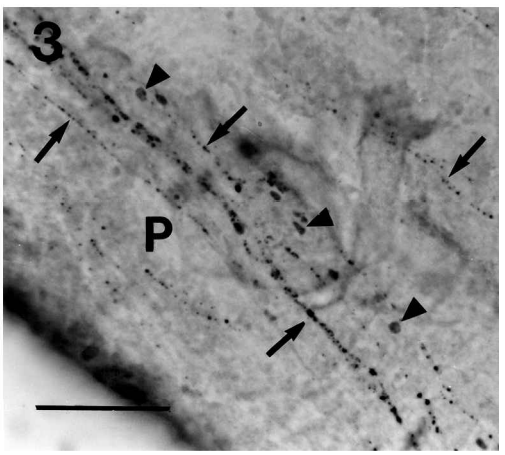
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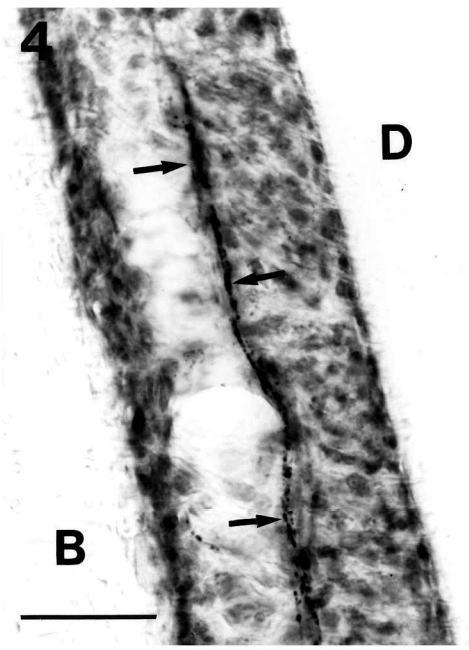
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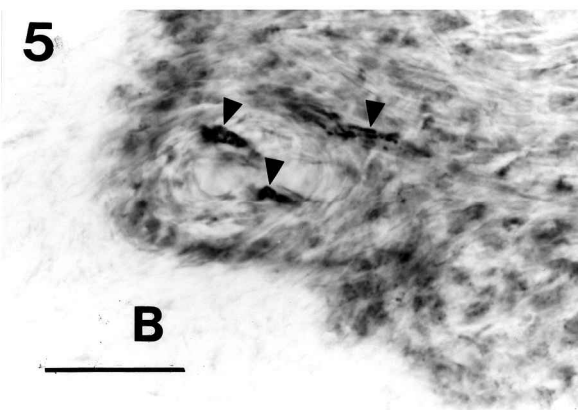
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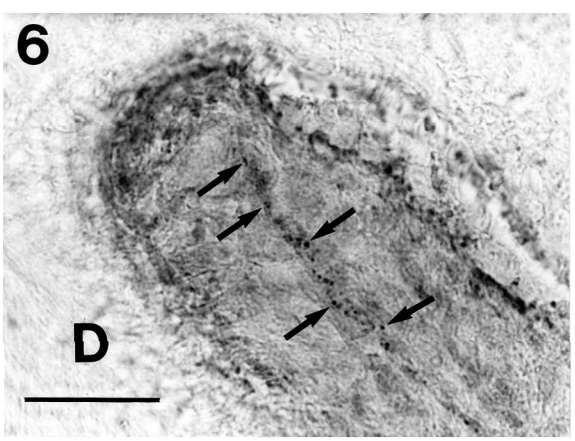
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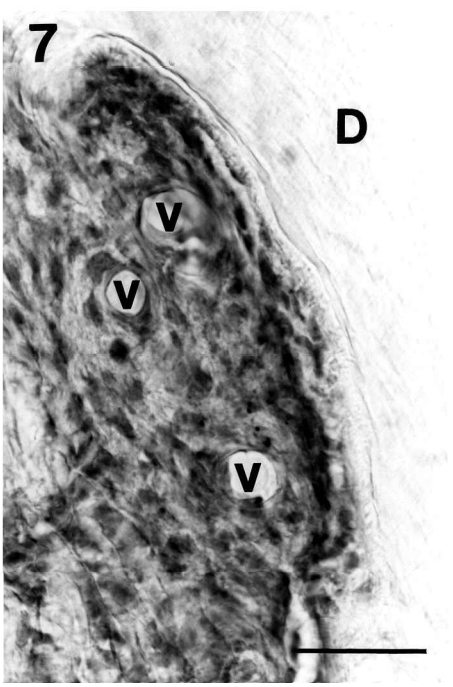
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Table 2. Measurements of hard-tissue responses in innervated and denervated replanted teeth

	Innervated teeth ($n = 12$)	Denervated teeth ($n = 12$)
Ankylosis	169.3 ± 49.7 μm	332.56 ± 193.2 μm (NS)
Root resorption	0.062 ± 0.029 mm^2	0.016 ± 0.0043 mm^2*

* $P < 0.02$; NS = not significant.

a Leitz light microscope at 10× to 100× magnifications. The evaluation of nerve fibers IR to SP and CGRP was done independently by two investigators. At least two slides for each antibody from all teeth were evaluated. The slides were coded to conceal their identity. The denervated side was consistently compared with the contralateral control side, to prevent interindividual differences.

Registration procedure

For the measurements of root resorption and ankylosis four central sections of the replanted teeth were used, in which the total length of the teeth was represented. The criteria for root resorption included both surface resorption (resorption lacunae on the root surface without inflammatory cells) and inflammatory resorption (resorption lacunae on the root surface containing inflammatory cells). Ankylosis was defined as replacement of periodontal membrane by bone, without cementum resorption or with cementum and dentin resorption.

The sections from the right and left mandible of each animal were measured with an image analyzer (Olympus AH 2 microscope) connected to CUE2 histomorphometric analyzing program (version 4.0; Galai Production, Migdal Haemek, Israel). Measurements were performed in 12 animals, and the values from each section were summarized and divided by the number of sections evaluated. Root resorption was calculated in area (mm^2), and ankylosis was measured in micrometers (Fig. 1). The Mann–Whitney test was used to compare the differences between paired data. Data are given as mean ± standard error of the mean.

Results

Nine ferrets had bifid first premolars, and five had single-rooted premolars. Single-root fractures were observed in six bifid premolars, and in one animal both roots were fractured. The latter and two other animals with root fractures on single-rooted teeth were excluded from the study. However, measurements of root resorption and ankylosis were only performed in roots that had not fractured.

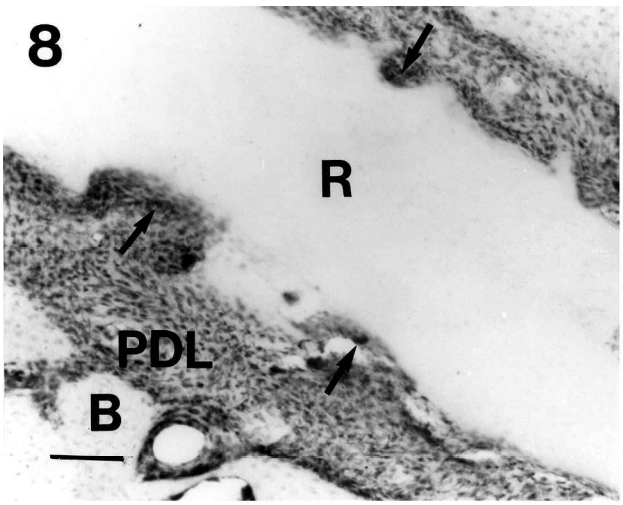
Dental pulp

Pulp necrosis was most frequently found on the denervated side (Table 1). Of a total of 12 denervated replanted teeth, only one showed a vital pulp without any necrotic areas. The vital pulps did not have a distinct odontoblastic layer, except in the most incisally part of some pulps, indicating that the pulp had regenerated from apical pulpal cells (17) or from cells in the apical periodontal ligament (18). In the regenerated vital pulps a few centrally located IR fibers for SP and CGRP were observed (Fig. 2). Partially necrotic pulps were observed in five replanted teeth on the innervated side, and in three of these, IR fibers were observed (Table 1). Mast cells, characteristically stained pink with methylene blue/azure II, were frequently observed in close proximity to the reinnervated IR fibers (Fig. 3) both in vital and in partially necrotic pulps.

Periodontal ligament

Six weeks after unilateral denervation all but one animal showed substantial reinnervation of the ipsilateral teeth and their supporting structures, including the periodontal ligament (PDL) of the replanted tooth (Fig. 4). Thin fibers IR to SP and CGRP could occasionally be observed close to resorptive areas along the bone side of the periodontal ligament (Fig. 5) and of the root surface (Fig. 6). SP-IR fibers were more frequently observed in resorptive lacunae than CGRP-IR. However, many resorptive areas were devoid of IR nerve fibers (Fig. 7). The controls with replacement of the primary or secondary antibody did not show any immunolabeling.

Fig. 8. Extensive bowl-shaped root resorptions (arrows) in a replanted tooth from the innervated side of the jaw. The section has been incubated without first antibody. B = bone; R = root; PDL = periodontal ligament. Bar = 100 μm.
Fig. 9. Areas with ankylosis (arrowheads) in the cervical part of the root of a replanted denervated tooth. No substance P fibers were observed in this section. B = bone; pdl = periodontal ligament; D = dentin. Bar = 100 μm
Fig. 10a. Micrograph of a root fracture in a replanted tooth from the innervated side of the mandible. R = root. Bar = 200 μm. 10b and c Higher magnification of outlined areas in 10a, showing bundles of calcitonin gene-related peptide-immunoreactive nerve fibers (arrowheads) between the fractured root surfaces. D = dentin. Bars = 50 μm.
Fig. 11. Section showing a root fracture in a replanted tooth from the denervated side. In the gap between the root surfaces newly formed bone is visible (arrows). Micrograph from a section immunolabeled for substance P but without any visible immunoreactivity. R = root. Bar = 100 μm.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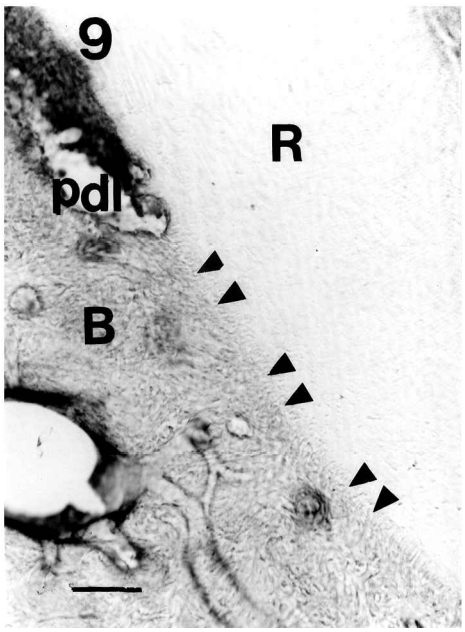
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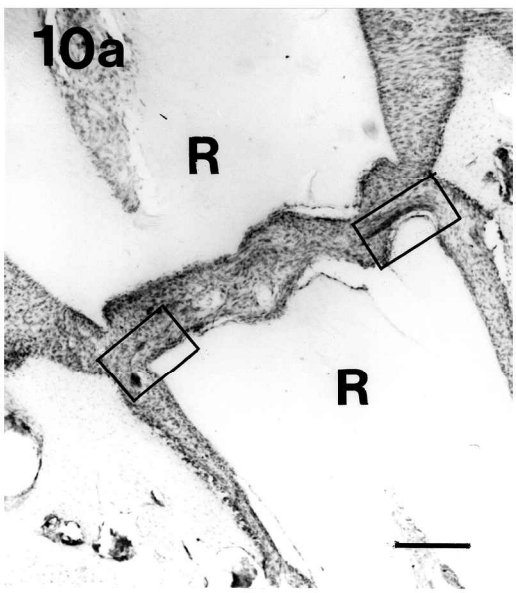
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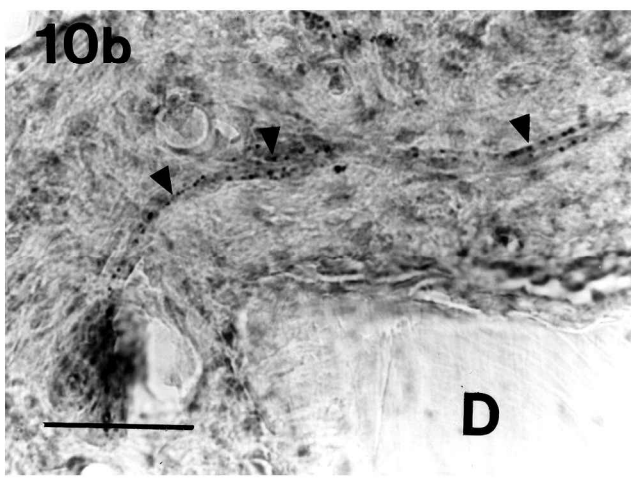
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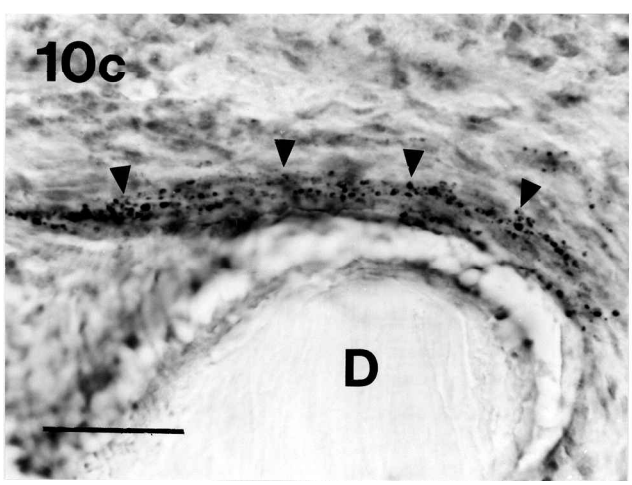
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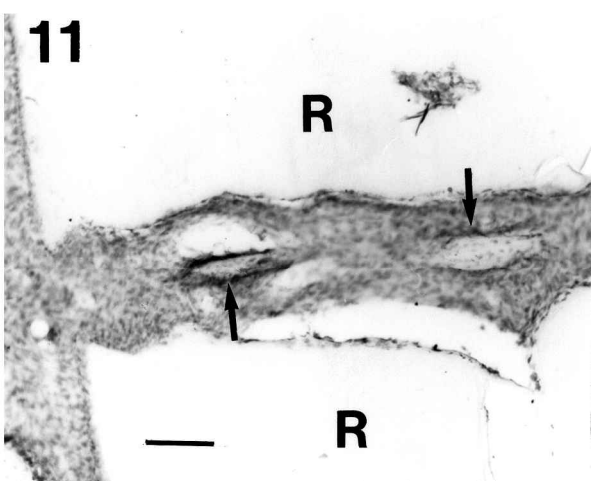
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Resorption and ankylosis

Some replanted teeth showed extensive inflammatory root resorption; these teeth did not have vital pulps. The resorbed areas were characterized by bowl-shaped cavitations (Fig. 8), often facing excavations in the adjacent bone. Despite considerably less frequent total pulp necrosis in the replanted teeth on the innervated side, the total root resorption areas were significantly greater than on the denervated side. Root resorption averaged 0.062 ± 0.029 mm^2 on the innervated side compared with 0.016 ± 0.0043 mm^2 on the denervated side. The difference was statistically significant ($P < 0.02$) (Table 2). External root resorption was observed in all replanted teeth ($n = 24$), whereas internal resorption was totally lacking. When ankylosis was present, islands of soft tissue with IR fibers could be traced between the ankylotic bone areas. Ankylosis was observed in four of the replanted teeth on the innervated side (169.3 ± 49.7 μm) and in six on the denervated side (332.56 ± 193.2 μm). The difference was not significant ($P = 1$) (Table 2). Ankylotic areas were usually observed on intact root surfaces and were rarely associated with root resorption (Fig. 9).

In areas of root fractures IR nerve fibers were observed between the fractured root-endings (Fig. 10). Bone formation, as evident in Fig. 11, could occasionally be traced in the gap between the fractured root endings.

Discussion

A main finding in this study was that IAN axotomy significantly reduced the root resorption areas of the replanted first premolar compared with the contralateral innervated control, indicating that nerves have an effect on osteoclast activation. Activation of osteoclasts in inflammatory bone destruction has previously been shown to be stimulated by cytokines interleukin (IL)-1α, IL-1β, and tumor necrosis factor (TNF) (19). Macrophages have been reported to be the main source of IL-1α, IL-1β, and TNF-α. A reduced number of macrophages has previously been found after injury in sensory denervated rat molars (20), whereas replantation of teeth in innervated jaws has implied an increased number of macrophage-associated antigen-expressing cells in the pulp (21). Hence, it might be assumed that deprivation of sensory neuropeptides in the denervated jaws might have caused reduced recruitment of macrophages and, consequently, less cytokine production and subsequent reduced osteoclast activation. SP increases osteoclastic activity both in vivo (22) and in vitro (12). However, this contrasts with observations by Toriya et al. (23), who found that the number of osteoclasts was reduced when the density of CGRP-IR nerve fibers reached a peak during the development of periapical lesions.

In the current study SP-IR nerve fibers were found more frequently in resorptive lacunae than CGRP-IR nerve fibers, but many resorption lacunae were without visible nerve fibers. In replanted teeth from dogs only a few PGP 9.5 IR nerve fibers entered the resorption lacunae 3 weeks after replantation (24), and no PGP 9.5 IR nerve fibers were present in the areas where root or bone resorption was in progress 2 weeks after replantation. These results indicate that the resorptional activity is independent of released neuropeptides acting directly on the osteosclasts, but intact nerve supply seems to enhance the resorptional activity in our study. Our observation is supported by a study by Adam et al. (25), who showed reduced osteoclastic bone resorption in mandibles of capsaicin-treated animals after tooth extraction in the ipsilateral maxilla compared with normal innervated controls. Vandevska-Radunovic et al. (26) observed reduced apical root resorption after axotomy of IAN in experimentally moved teeth in rats. Application of mechanical stimuli to teeth and their supporting tissues induces a cascade of inflammatory responses. Therefore, their results are in line with those of the current study and indicate that the development of inflammatory reactions is partly dependent on the existence of intact sensory nerve supply. A previous study has shown a decrease in blood flow in the ferret dental pulp after axotomy of IAN (27), and other studies have shown that the neuropeptides CGRP and SP exert a vasodilatatory effect on vessels in both the dental pulp and gingiva (28, 29). Impaired blood flow after axotomy may also occur in the PDL, and this may contribute to the difference in healing responses observed after replantation of teeth. An assumed reduced blood flow in the PDL after axotomy can induce a restrained inflammatory response with fewer blood-born immunocompetent cells in the replantation area than in the innervated side.

In this study no significant difference between the innervated and the denervated side was found with regard to ankylosis, which may indicate that osteoblastic activity is mainly regulated by factors other than sensory nerves. The result supports the findings of Hill et al. (30), who observed no significant difference in tibia cortical, medullary, or periosteal bone apposition rates after capsaicin treatment in neonatal rats, compared with controls. Andreasen (16) found that a high number of damaged cementoblasts after replantation led to healing with ankylosis. As long as the treatment of replanted teeth was similar on both the innervated and denervated side in the current study, there would be no reason to expect the number of damaged cementoblasts to differ on the two sides.

Pulpal necrosis developed in 10 denervated replanted teeth, compared with five in the innervated side. This result indicates that the innervation in the periapical area may promote survival of the pulp tissue in the replanted teeth. This observation might be explained by the stimulatory effect on angiogenesis induced by sensory neuropeptides (8). However, the difference can also be due to the already discussed fall in blood flow measured after denervation. In teeth with root fractures, nerve fibers IR for CGRP and SP that ran between the fractured ends were frequently observed. This observation is in line with studies in bone fractures, in which especially CGRP-IR fibers seemed to sprout in the area of trauma (31). This

sprouting may have a potential importance in the vascular control of fracture, angiogenesis, and osteogenesis, in addition to a protective effect against excessive fracture movement. In teeth the latter can warn against and protect from direct biting during healing after a root fracture.

It is concluded that the sensory nerves promote root resorption after pulpoperiodontal injuries, but they have less influence on the osteoblastic activity expressed by ankylosis. This study also indicates that an intact nerve supply in the jaw helps to promote survival of the pulp after replantation.

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