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Bisphosphonate Uptake in Areas of Tooth Extraction or Periapical Disease

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Abstract

Purpose—Bisphosphonates (BPs) are widely used for the management of bone diseases such as osteoporosis and bone malignancy. However, osteonecrosis of the jaws (ONJ) is a serious complication of BP treatment. ONJ lesions mainly occur after extraction of teeth deemed unrestorable or around teeth with active periodontal or periapical disease. Because socket healing or dental disease shows higher bone turnover, the authors hypothesized that preferentially high BP accumulation would be observed in these areas.

Materials and Methods—The authors tested the uptake of fluorescein-labeled zoledronic acid (5-FAM-ZOL) in sites of tooth extraction or experimental periapical disease in mice. Maxillary molars were extracted or the crowns of mandibular molars were drilled to induce pulp exposure. Animals were injected with 5-FAM-ZOL 200 μg/kg at various times after intervention and fluorescence was measured at healthy versus intervention sites. Fluorescein injections were used as controls. Data were analyzed by *t* test and mixed effects linear models were constructed because the animals had repeated measurements over time and at the 2 sites.

Results—A statistically significant ($P \leq .001$ to .002) time-dependent uptake of 5-FAM-ZOL was detected in the areas of extraction socket and in the alveolar ridge around teeth with periapical

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disease compared with the healthy contralateral sites of the same animals. For the 2 conditions, the uptake reached a maximum 3 days after experimental intervention and decreased thereafter.

Conclusions—These data suggest that sites with increased bone turnover, such as extraction sites or areas of periapical inflammation, are exposed to higher BP doses than the remaining alveolar ridge and could explain, at least in part, the susceptibility of such areas to ONJ.

Medication-related osteonecrosis of the jaws (ONJ) is a serious complication of treatment with antiresorptive or antiangiogenic agents, chiefly affecting patients with cancer or metabolic bone disease.[1] ONJ refers to exposed necrotic bone in the maxillofacial region for longer than 8 weeks in patients with current or previous BP treatment and without a history of radiation therapy to the jaws. ONJ incidence and severity correlate with dose, mode of administration, and duration of treatment and are typically observed with high-potency, high-dose, nitrogen-containing BPs, such as zoledronate given intravenously.[1-3]

A puzzling question is the nearly exclusive selection of the disease for the jaws, although the remaining skeleton appears to be spared. Several hypotheses for this increased predilection have been proposed. The maxilla and mandible are close to the external environment lined by stratified squamous epithelium with only a narrow layer of underlying lamina propria.[4] Thus, injury of the thin oral mucosa would readily expose the underlying bones to the oral cavity.[5,6] In addition, oral tissues are frequently exposed to bacterial infection through periodontal and periapical disease. Immune response is vital in defending these infectious processes and BP treatment has been associated with alterations in immune cell function.[7]

Cells of the mandible and maxilla might differ from those of other bones. The jaws are derived from neural crest cells and undergo intramembranous instead of endochondral ossification.[8] Differences between mandibular and long bone osteoblastic and osteoclastic proliferation, differentiation, and function have been reported.[9-12] Ovariectomy or malnutrition affect mandibular versus tibial trabecular architecture and bone mineral density differently.[13] Importantly, basal homeostasis of mandibular and maxillary bones might be increased compared with the remaining skeleton.

Intracortical remodeling rate in the jaws versus iliac crest has been reported to be 10 to 20 times higher in humans or animals.[5] This increased jawbone metabolism would result in accumulation of high BP levels to the jawbone matrix and could compromise function and differentiation of bone cells in the maxilla and mandible. Conversely, bone scintigraphic studies have found that radionuclide uptake, reflecting bone turnover, is similar for the mandible and femur and considerably lower than for the maxilla.[14-16] Thus, whether jaws display increased bone turnover compared with the remaining skeleton and whether such differences play a role in ONJ pathogenesis remain controversial.[17]

Most ONJ lesions occur after extraction of teeth deemed unrestorable owing to caries or around teeth with active periodontal or periapical disease.[3,18] The authors hypothesized that bone injury and healing after extraction or bone infection or inflammation from periapical disease would increase local alveolar bone deposition of fluorescein-labeled zoledronate (5-FAM-ZOL) in mice. The present findings showed an important time-dependent accumulation of the labeled drug in sites of tooth extractions or dental disease and suggested

that sites of increased risk for ONJ development are subjected to higher BP exposure compared with healthy sites with basal bone homeostasis.

Materials and Methods

Animal Care and Tooth Extraction or Experimental Periapical Disease Induction

Twenty-seven 16-week-old C57BL/6J male mice (Jackson Laboratories, Bar Harbor, ME) were used. All animals and surgical procedures were handled according to guidelines of the chancellor's animal research committee of the University of California–Los Angeles. All animals recovered well from the experimental procedures without any obvious adverse effects.

Animals were anesthetized with isoflurane. For tooth extraction, the left maxillary first and second molars were removed, and the right maxillary molars were kept intact. For experimental periapical disease, the right first and second mandibular molar crowns were drilled to induce pulp exposure, avoiding furcal perforation,[19,20] and the left molars were kept intact.

5-FAM-ZOL was synthesized by the conjugation of 5-carboxyfluorescein with zoledronate functionalized by an epoxide linker. The product was purified by high-performance liquid chromatography and fully characterized by high-performance liquid chromatography, ultraviolet-visible spectroscopy and fluorescence-emission spectroscopy, ^{1}H and ^{31}P nuclear magnetic resonance spectroscopy, and high-resolution mass spectoscopy.[21,22] Animals received intraperitoneal (IP) injections of 5-FAM-ZOL 200 μg/kg. Four negative control animals underwent tooth extractions or molar drilling and received IP injections of 5-carboxyfluorescein 200 μg/kg.

Micro-Computed Tomographic Scanning

Maxillas and mandibles were removed en bloc, fixed in 4% paraformaldehyde for 48 hours, and kept in the dark. Bones were imaged by micro-computed tomographic (μCT; SkyScan 1172; SkyScan, Kontich, Belgium) scanning at 10-μm resolution. Volumetric data converted to Digital Imaging and Communications in Medicine format were visualized using Dolphin Imaging software (Chatsworth, CA).[19]

Fluorescence Imaging

Soft tissues were carefully removed. Maxillas were positioned on the platform with the palatal side of the alveolar ridge facing the camera. The hemimandibles were dissociated and the left and right sides were placed with the buccal surface toward the platform and the lingual surface toward the camera.

Fluorescence imaging (CRi Maestro2, Woburn, MA) at 520-nm wavelength and 25-ms exposure was performed. The fluorescein isothiocyanate filter was selected, with acquisition start-to-end wavelengths of 500 to 720 nm in 10-nm stepwise increments. To acquire the highest level of resolution, 1 × 1 binning was selected for all imaged bones. Separate focus levels were selected for the maxilla and the mandible to correct for the different anatomies. Fluorescence data were converted to CRi Cube (.im3) format and analyzed with Maestro

software. Fluorescence signal was measured around the maxillary or mandibular molars and was normalized to the region of interest. Regions of interest covered the same area for all mandibles or maxillas, but were different between the 2 bones.

Statistics

Mean and standard error of mean were computed using Excel (Microsoft, Redmond, WA). Fluorescence intensities were transformed using a $\log(x + 1)$ transformation to decrease the skew in their distributions. Intensities were compared between control and treated animals using *t* tests and were compared between sites (healthy vs intervention site) of the treated animals using paired *t* tests. Mixed effects linear models were constructed to evaluate the effects of site, time, and site-by-time interaction. Mixed effects models were used because animals had repeated measurements over time and at the 2 sites. Statistical analyses were performed using SAS (SAS Institute, Cary NC).

Results

Experimental Protocol

One, 3, 5, or 7 days after tooth extraction or pulp exposure (Fig 1, arrows), animals received 5-FAM-ZOL injections (Fig 1, arrowheads). Forty-eight hours after injection, animals were euthanized (Fig 1, vertical line). Each group contained 5 to 8 animals. Five additional animals without any intervention received the same 5-FAM-ZOL IP injections and were sacrificed 48 hours later (Fig 1, control).

Successful interventions were assessed by, μCT scanning. Figure 2A shows, μCT images of the maxilla from an animal that underwent extractions 7 days before 5-FAM-ZOL injections. Initiation of socket healing can be observed on cross-sectional, sagittal, and axial slices. Figure 2B depicts the healthy and drilled mandibular first and second molars of a mouse 3 days before 5-FAM-ZOL injections. Widening of the periodontal ligament space can be appreciated on the coronal and sagittal slices, whereas axial sections show loss of alveolar bone at the furcation level of the drilled molars.

5-FAM-ZOL Uptake in Healthy and Extraction Areas of the Maxillas

Dissected maxillas were positioned with the palatal surface facing the camera (Fig 3A) and fluorescence was measured. Low-level fluorescence was seen in the control animals. Tooth extractions resulted in markedly increased fluorescence of the alveolar ridge that peaked at 3 days after extraction and decreased thereafter, but remained higher than at the healthy nonextracted site (Fig 3A). A slight increase in fluorescence was observed in the alveolar bone around nonextracted teeth for 1 and 3 days after extraction (Fig 3A). No fluorescence was detected in extraction sites of animals injected with 5-carboxyfluorescein (not shown). Quantitation of fluorescence (Fig 3B) showed a statistically important increase for the extraction site at days 1 (55-fold over control and 11.7-fold over healthy site), 3 (84.5-fold over control and 32-fold over healthy site), 5 (16.3-fold over control and 32.4-fold over healthy site), and 7 (3.15-fold over control and 14.3-fold over healthy site). A significant ($P < .001$) site effect (intervention greater than healthy) over time, significant ($P < .001$) time effects (decrease in fluorescence over time), and asignificant ($P = .002$) site-by-time

interaction (intervention sites decreased more rapidly over time than did healthy sites) were found.

5-FAM-ZOL Uptake in Mandibular Alveolar Ridge of Healthy or Drilled Molars

Dissected hemimandibles were positioned with the lingual surface of the alveolar ridge facing the camera (Fig 4A) and fluorescence was measured. Control untreated animals showed low-level fluorescence. Experimental periapical disease, induced by crown drilling and pulp exposure, increased fluorescence of the alveolar ridge that was maximal at 1 and 3 days after drilling and decreased at later time points, but remained higher than at healthy sites (Fig 4A). A slight increase in fluorescence was seen in the alveolar ridge of healthy teeth at 1 and 3 days after drilling of the contralateral site molars (Fig 4A). No fluorescence was detected in the extraction sites of animals injected with 5-carboxyfluorescein (not shown). Quantitation of normalized fluorescence counts (Fig 4B) showed a statistically important increase for the alveolar bone at the drilled site at days 1 (39.1-fold over control and 14.3-fold over healthy site), 3 (33-fold over control and 12.8-fold over healthy site), and 5 (6.5-fold over control and 5.4-fold over healthy site). A slight but statistically important increase for the alveolar bone at the healthy site over control at day 3 (2.6-fold) was noted. A significant (P = .001) site effect (intervention greater than healthy) over time, significant (P = .02) time effects (decrease in fluorescence over time), and a significant (P = .03) site-by-time interaction (intervention sites decreased more rapidly over time than did healthy sites) were found.

Discussion

Although ONJ has been reported for nearly a decade, the pathophysiologic mechanisms of the disease remain largely unknown.[3] ONJ is most frequently observed after tooth extraction as a failure of extraction socket healing and resultant bone exposure.[2,3] Teeth in adults are removed when deemed unrestorable owing to severe caries or periapical or periodontal disease.[23,24] Thus, extractions and dental disease are the most frequent dental predisposing factors of ONJ.

A puzzling conundrum is the nearly exclusive incidence of the disease to the jaws, whereas the remaining skeleton is spared.[5,7] Various hypotheses have been proposed to explain the jaw predilection to osteonecrosis in the presence of BPs.[1] Among them, increased bone turnover of the jaws compared with the remaining skeleton and oversuppression of bone turnover by antiresorptive medications suggest a preferential uptake of BPs to the maxilla and mandible.[1,5]

Methylene bisphosphonic acid (medronic acid [MDP]), the parent form of BP, binds with high affinity to bone mineral. For this reason, technetium-99m (^{99m}Tc)-labeled MDP is used in bone scans for localization of sites with high bone turnover in diseases such as bone malignancy or inflammation.[25] Bone scans of patients with breast or prostate cancer obtained to evaluate bone metastasis were retrospectively evaluated to explore whether basal bone homeostasis in the maxilla and mandible were different than the remaining skeleton.[14,15] In the 2 patient cohorts, the investigators reported that radionuclide uptake was similar between the mandible and femur and considerably lower than that of the

maxilla. Interestingly, ONJ affects the mandible more frequently than the maxilla[1]; thus, if basal bone remodeling had played a key role in ONJ pathogenesis, radio-nuclide uptake would be expected to have been higher in the mandible.[17] The investigators concluded that increased BP uptake and bone turnover oversuppression are unlikely to play a major role in ONJ pathogenesis.[14-16]

Although the role of basal bone homeostasis in jaw sensitivity to osteonecrosis remains to be determined,[17] localized heightened, and not baseline, bone turnover could explain the preferential accumulation of BPs to alveolar sites at risk for developing ONJ. Indeed, local factors associated with ONJ risk, such as oral surgical procedures, dental infection, and poor-fitting dentures,[1] induce inflammatory changes, stimulate osteoclastic and osteoblastic activity, and increase bone turnover at such sites.[26,27]

In the present study, the authors explored the potential preferential accumulation of BPs in localized areas of the alveolar ridge during socket healing or dental infection or inflammation using 5-FAM-ZOL as an imaging probe. They used well-characterized mouse models of tooth extraction[28] and experimental periapical disease,[19] in conjugation with 5-FAM-ZOL, a potent nitrogen-containing BP used commonly for the management of malignant bone disease or osteoporosis and the BP most commonly associated with ONJ.[3] 5-FAM-ZOL retains important biochemical and biologic functions of the parental compound. 5-FAM-ZOL binds with high affinity to synthetic calcium phosphate–coated discs, with binding characteristics that follow the classic Langmuir adsorption isotherm.[29] Importantly, 5-FAM-ZOL retains approximately 50% of the parent drug's anti-resorptive activity in vivo and appreciably increases trabecular bone volume and structural parameters in the long bones of rats deficient in vitamin D.[29]

Fluorescently labeled BPs have been used to characterize the biochemical properties of various drugs, their biodistribution in bone sites, and the biologic response of bone cells to them. Risedronate is a nitrogen-containing BP used primarily in the management of osteoporosis.[30] Carboxyfluorescein- and Alexa Fluor 647–labeled risedronate have been used to visualize BP binding to bone mineral in trabecular and intracortical drug distribution, localization in resorbing and forming surfaces, around newly embedded osteocytes, and uptake by osteoclasts and monocytes.[31-33] FAM-labeled risedronate has been used to visualize increased BP internalization by osteoclasts from mandibular versus long bone marrow and higher levels of prenylation inhibition in mandibular osteoclasts.[34] Moreover, 5-FAM-ZOL has been used to evaluate long bone and mandible adsorption of zoledronic acid administered as a single injection versus repeated injections.[29]

The present data indicate that 5-FAM-ZOL preferentially accumulates in sites of tooth extractions or dental disease compared with healthy sites. These areas of increased bone turnover are exposed to higher BP than the remaining alveolar ridge and thus would be more susceptible to the drug effects. Furthermore, these data may explain, at least in part, the occurrence of ONJ at sites of previous extractions or around teeth with severe dental disease.[3,18] Importantly, a time-dependent fluorescent signal was observed at the area of periapical disease or tooth extraction, with high levels soon after experimental intervention and decreasing thereafter. From a clinical perspective, these observations suggest that high

drug levels would accumulate at the extraction site in a patient receiving BPs shortly after tooth extraction. However, a delay in BP administration would result in lower drug levels at the extraction site that might cause a minor disturbance in wound healing.

The authors also noted a small increase in fluorescence uptake of the healthy maxillary or mandibular site at 1 and 3 days that did not reach statistical significance ($.05 < P < .1$), except for the mandible at 3 days after drilling. From a clinical viewpoint, this unexpected observation implies possible increased BP uptake in healthy areas of the alveolar ridge, when adjacent sites show increased bone turnover.

These experimental findings are supported by clinical observations. Bone scans performed in patients for skeletal surveys have indicated increased ^{99m}Tc-MDP radionuclide uptake at sites of healing extraction sockets, periapical and periodontal disease, and ill-fitting dentures,[35,36] reflecting localized increase bone remodeling and suggesting that systemically administered BPs would preferentially accumulate at high levels in such areas of the jaws.

In conclusion, these data support a time-dependent ZOL deposition at sites of tooth extraction or around teeth with periapical disease. This preferential localized alveolar ridge accumulation suggests that increased bone turnover at sites prone to develop ONJ, and not basal maxilla or mandible homeostasis, might be an important determinant of the increased jaw sensitivity to osteonecrosis.

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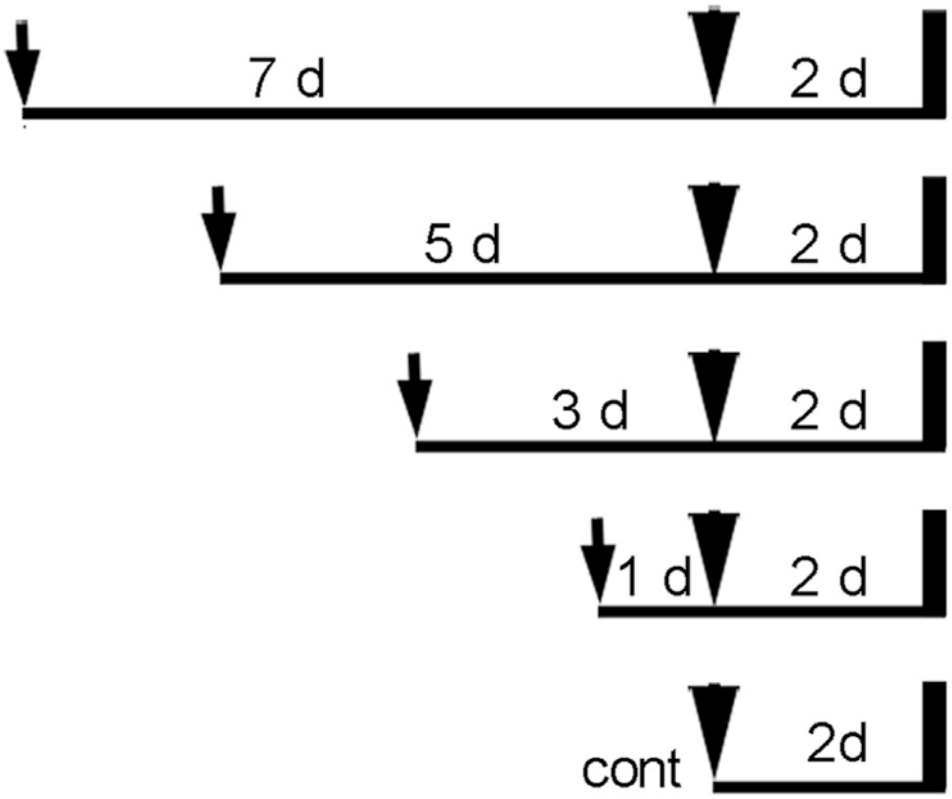

Figure 1.
Diagram for experimental design: surgical intervention (extraction or tooth drilling; *arrows*), fluorescein-labeled zoledronic acid injection (*arrowheads*), and time of sacrifice (*vertical lines*).

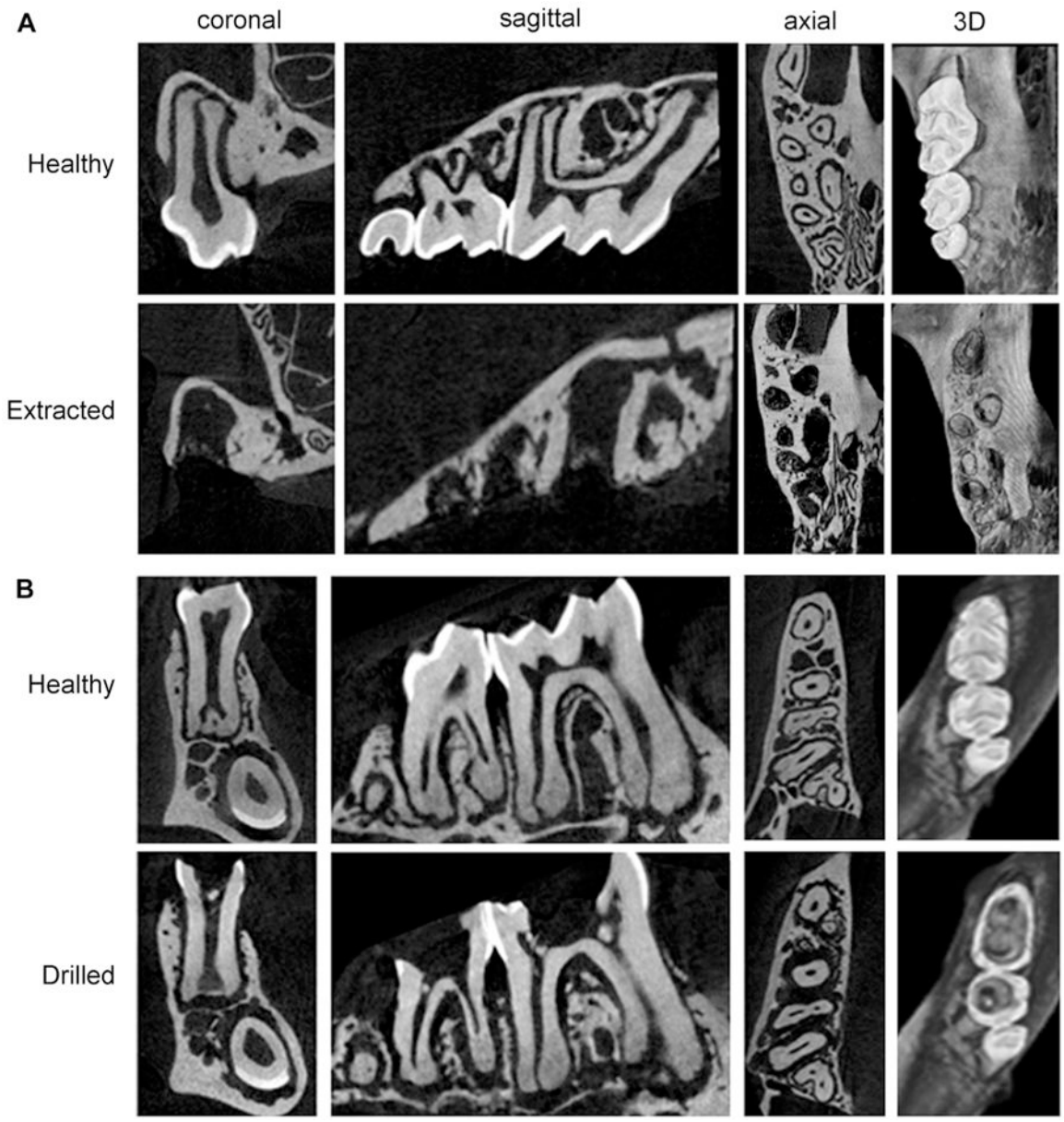

Figure 2.

Coronal, sagittal, and axial slices and 3D reformatted images of the *A*, maxillary alveolar ridge at the healthy and extraction sites and *B*, the mandibular alveolar ridge at the healthy and crown drilled sites. 3D, 3-dimensional.

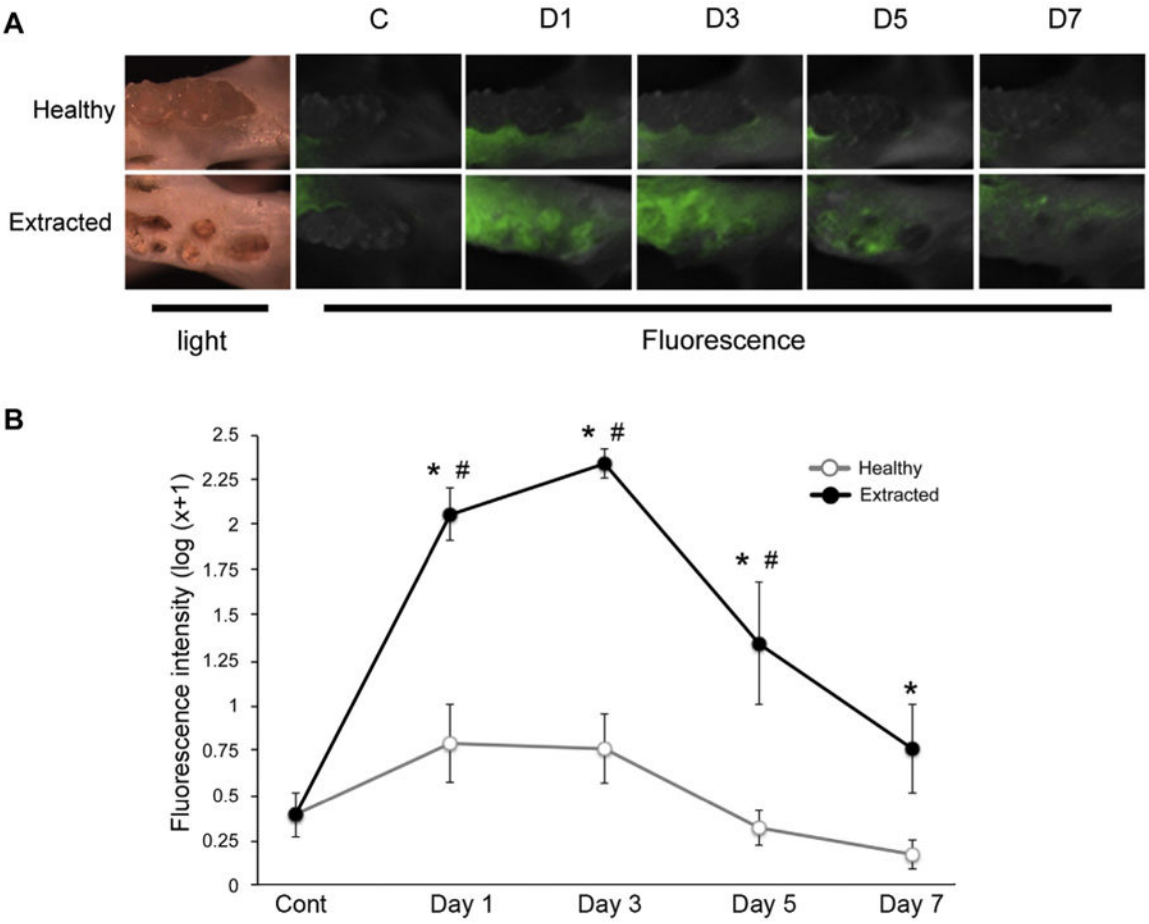

Figure 3.

A, Light and fluorescence imaging of healthy and extraction sites of the maxillary alveolar ridge at 1, 3, 5, and 7 days after extraction. Control animals did not receive extractions. *B*, Quantification of fluorescence expressed as log(x + 1). *Statistically significant from healthy sites in the same animals; #statistically significant from untreated controls (P < .05). C, control; D1, 1 day; D3, 3 days; D5, 5 days; D7, 7 days.

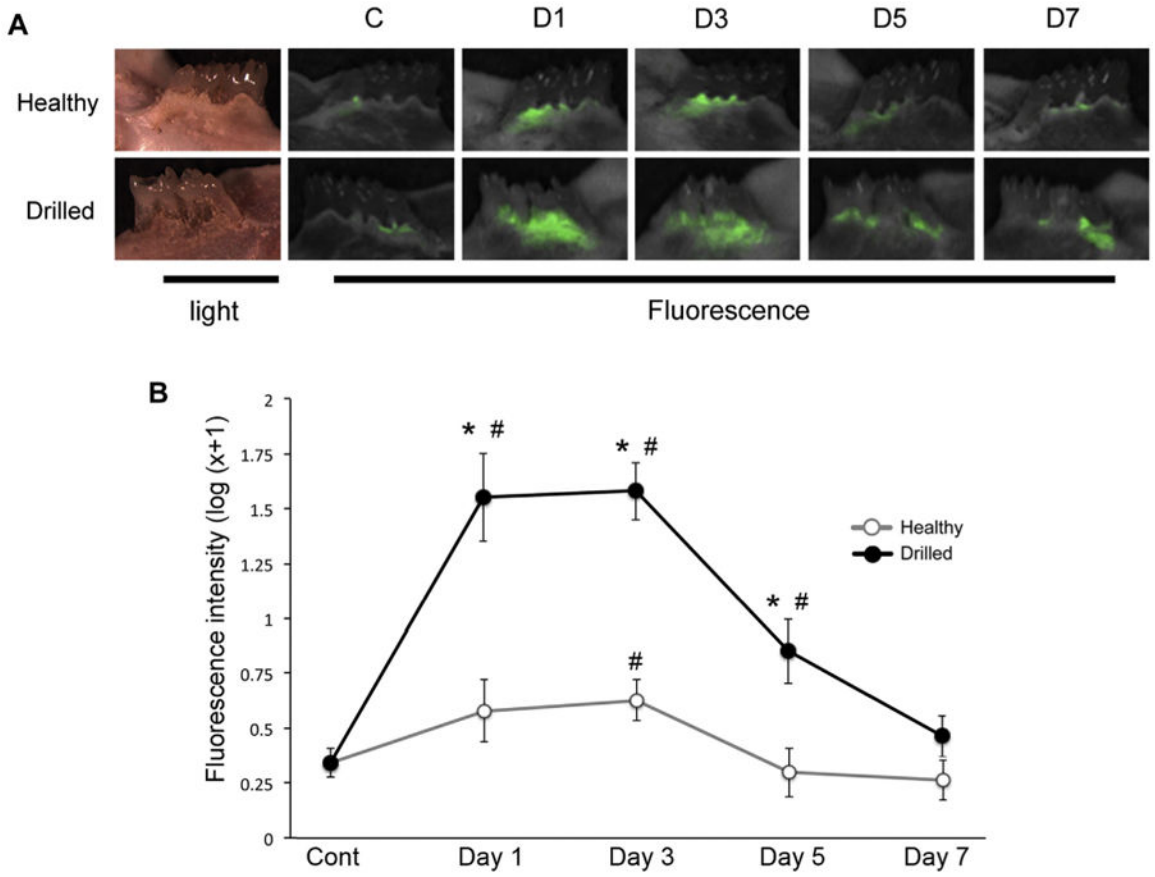

Figure 4.

A, Light and fluorescence imaging of the healthy and drilled sites of the mandibular alveolar ridge at 1, 3, 5, and 7 days after crown drilling. Molar crowns of control animals were not drilled. *B*, Quantification of fluorescence expressed as log(x + 1). *Statistically significant from healthy sites in the same animals; #statistically significant from untreated controls (P < .05). C, control; D1, 1 day; D3, 3 days; D5, 5 days; D7, 7 days.