Impact of nicotine on bone healing

Jeffrey O. Hollinger,[1] John M. Schmitt,[1] Kun Hwang,[1] Peiman Soleymani,[2] Dave Buck[1]
[1]*Northwest Wound Healing Center, Division of Plastic and Reconstructive Surgery, School of Medicine, Oregon Health Sciences University, Portland, Oregon 97201-3098*
[2]*Department of Periodontology, School of Medicine, Oregon Health Sciences University, Portland, Oregon 97201-3098*

Received 21 October 1998; accepted 10 November 1998

Abstract: A limited number of experimental animal studies and *in vitro* data confirm that nicotine impairs bone healing, diminishes osteoblast function, causes autogenous bone graft morbidity, and decreases graft biomechanical properties. Therefore, our long-term goal is to develop an effective therapy to reverse the adverse impact of nicotine from tobacco products. However, before accomplishing this goal, we had to develop an animal model. Our hypotheses were nicotine administration preceding and following autogenous bone grafting adversely affected autograft incorporation and depressed donor site healing in a characterized animal wound model. Hypothesis testing was accomplished in bilateral, 4-mm diameter parietal bone defects prepared in 60 Long–Evans rats (male, 35-day-old). A 4-mm diameter disk of donor bone was removed from the left parietal bone and placed in the contralateral defect. The donor site served as a spontaneously healing bone wound. The rats were partitioned equally among three doses of nicotine administered orally in the drinking water (12.5, 25, and 50 mg/L). For each dose, the duration and sequence of nicotine treatment followed four courses, including no nicotine and designated combinations of nicotine administration and abatement prior to and following osseous surgery. Experimental sites were recovered on 14 and 28 days postsurgery, responses quantitated, and data analyzed by analysis of variance and *post hoc* statistics ($p \leq 0.05$). We developed a convenient and effective osseous model, and the results validated our hypothesis that nicotine negatively impacts on bone healing. © 1999 John Wiley & Sons, Inc. J Biomed Mater Res, 45, 294–301, 1999.

Key words: nicotine; parietal bone wound model; bone healing; autograft

INTRODUCTION

Autografts are the preeminent choice to correct osseous craniomaxillary and mandibular defects because of the relatively high predictability for a successful outcome.[1] However, for patients who use tobacco and receive autogenous bone grafts, the success rates plummet.[2–4] A 15 patient clinical study revealed that 80% of the individuals with impaired osseous healing were smokers.[5] In another clinical study of more than 400 patients, localized alveolitis delayed osseous healing of dental extraction sites at a level 4–5 times greater for smokers than nonsmokers.[6] Furthermore, after spine fusion with bone autografts, pseudoarthrosis was 4 times more prevalent in patients who smoked than those who did not smoke.[2,3,7] Hadley and Reddy[8] reviewed 12 separate clinical studies confirming similarly alarming statistics.

Nicotine in tobacco products causes peripheral vasoconstriction[9] and tissue ischemia (reviewed by Jones and Triplett[5]) and decreases oxygen tension.[10] Moreover, nicotine depresses osteoblast activity.[3,4,11–14] Therefore, we and others reason that nicotine is the main pathophysiological factor in tobacco products that causes bone graft morbidity and impaired osseous healing.[13,14]

These observations, as well as clinical reports, have prompted clinicians to inform patients receiving bone grafts to refrain from tobacco products prior to and following surgery. The period of time preceding and succeeding grafting is often arbitrary. Moreover, the noncompliant patient who uses tobacco products and is to receive a bone graft presents a daunting clinical challenge.

Our long-term goal is to develop an effective therapy to reverse the adverse impact of nicotine-induced peripheral vasoconstriction from tobacco products. To accomplish this goal, we first had to develop a convenient, economical, and reliable animal wound model. A bilateral model in parietal bones was developed that included a spontaneously healing wound and a recipient site for an autograft. Therefore, within the same animal, responses to nicotine dosing

Correspondence to: Dr. J. O. Hollinger; e-mail:hollinge@ohsu.edu

 CCC 0021-9304/99/040294-08

Nicotine Administration Timeline

	Days Prior to Surgery		Days after Surgery		
Treatment Groups*	28 -14	13 -1	0	0 - 14	15 - 28
Group 2	■		Surgery		■
Group 3	■	■	Surgery		■
Group 4	■	■	Surgery	■	■

*Note: Group 1 did not receive any nicotine and groups 2-4 were each subdivided into three sub-groups which were administered 12, 25, and 50 mg/L nicotine for each timepoint indicated.

Key:
□ No Nicotine
■ Duration of Nicotine Administered

Figure 1. Table indicating the dosing and timing for nicotine administration.

and timing could be determined at a bone-healing donor site and at the autograft recipient bed. Using established morphological quantitative methodology, we determined nicotine administration deters osseous regeneration within a spontaneously healing wound.

MATERIALS AND METHODS

Experimental design

Bilateral, 4-mm diameter parietal bone defects were prepared in 60 35-day-old male Long–Evans rats. The rats were partitioned equally to receive one of three doses (12.5, 25, and 50 mg/L) of nicotine (Sigma Chemical Company, St. Louis, MO) administered in the drinking water as previously described.[12,15] The time line for administration of nicotine is presented in Figure 1. Each group had six rats. The determination of this sample size among treatments and across times was accomplished through power analysis (at 0.90).[16,17]

Surgery

Rats were placed in an anesthesia box with 3% isoflurane until a surgical plane of anesthesia was obtained. The sur-

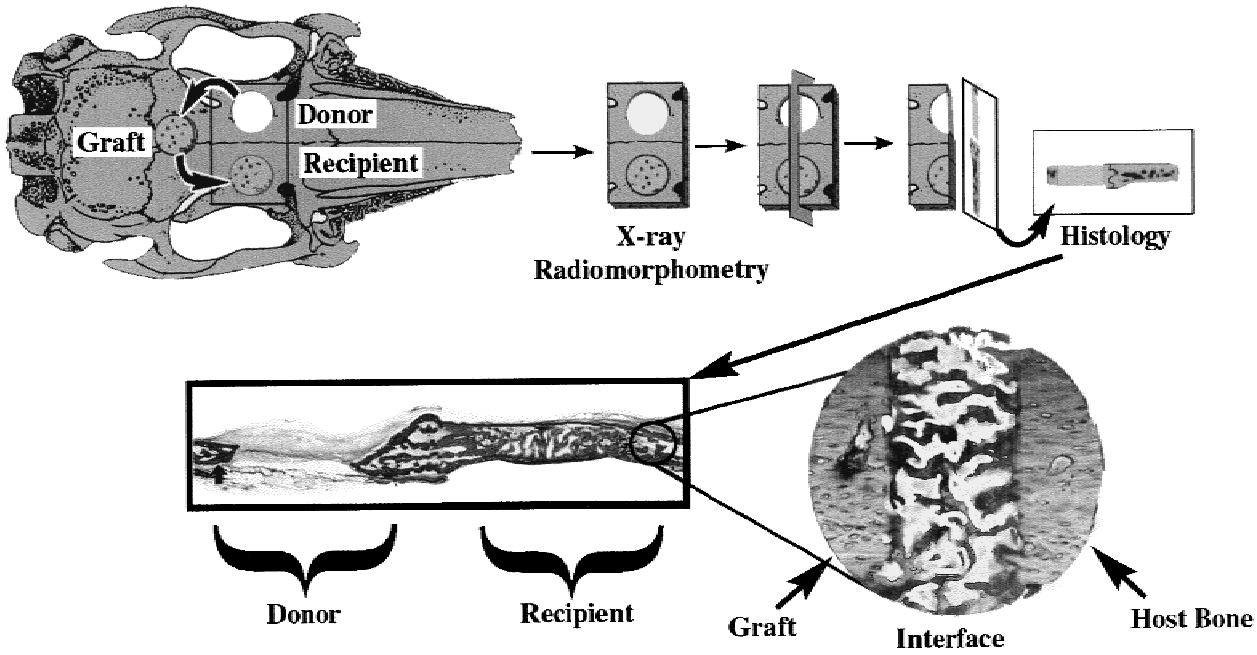

Figure 2. Schematic with donor and recipient parietal bone defects and progression of events including tissue retrieval, processing, and graft–host interface observation.

gical site was prepared for aseptic surgery and Lacri-lube® (Allergan Pharmaceuticals, Allergan Inc., Irvine, CA) ophthalmic ointment was placed gently onto the conjunctiva of each eye.

A linear incision was made from the posterior occipital protuberance to the nasal bone, the periosteum was elevated, and a 4-mm diameter craniotomy was prepared in each parietal bone between the temporal line and the midsagittal suture. The procedure was accomplished with a surgical trephine, Bell hand piece, and physiological saline irrigation as previously described.[18]

The left craniotomy provided donor material that was inverted (i.e., dural side cephalic) and implanted into the right craniotomy. The left donor site was the spontaneously healing bone wound, while the right side contained the healing recipient autogenous graft (Fig. 2).

Soft tissues were closed with absorbable 4-0 Biosyn (USSC, New Haven, CT) sutures. Each rat was monitored closely postoperatively and received a veterinary analgesic for routine postoperative pain control.

Animal experimentation was conducted in accordance with guidelines for animal care and use promulgated by the National Institutes of Health (publication 85-23, revision 1985), as well as the institutional animal care and use committee at Oregon Health Sciences University (Portland, OR).

Radiomorphometry

Following necropsy at the designated times, experimental sites (i.e., specimens) were recovered and placed in 70% ethanol. After 24 h, specimens were radiographed in a standard manner using a Minishot X-ray cabinet (Associated X-ray Co., East Haven, CT) with a constant object to film distance and ultrahigh contrast mammography film (X-OMATL, Kodak, Eastman Kodak, Rochester, NY). X-ray films of the specimens were assessed in a standardized fashion with gray level densities using an image analysis system (Leica Instruments Ltd., Cambridge, UK) as previously reported.[19–21] Radiopacity at the spontaneously healing wound and the autograft recipient site was expressed as a mean percentage and 1 standard deviation.

Histology and histomorphometry

Experimental sites were recovered *en bloc* and placed immediately in 70% ethanol, taken to 100% ethanol, and embedded in poly(methylmethacrylate). Following curing, embedded samples were cut to 4.5–5 μm thick sections using a microprocessor controlled Polycut E microtome (Leica Instruments Ltd., Cambridge, UK). Sections were mounted onto glass slides with 100% ethanol, pressed, and warmed overnight at 60°C for staining with a modification of the Goldner–Masson trichrome stain and von Kossa stain.

Goldner–Masson trichome-stained sections were examined with bright field light microscopy for cell type, morphology, and stromal detail with a Zeiss Axiophot Microscope (Zeiss Instruments Co., Inc., New York). Von Kossa stained specimens were quantitated for new bone within the spontaneously healing donor site with an image analysis system and Zeiss Axiophot microscope and reported in square millimeters as previously described.[19–21] The new bone in the autograft recipient site was measured in a similar manner at the interface between the host bone and the autograft (Fig. 2). The resultant value for each treatment group reported was expressed as the mean in square millimeters and 1 standard deviation.

The data for the percent radiopacity and square millimeters of bone formation were analyzed by an analysis of variance (ANOVA) and Fisher's protected least significant difference test to detect differences between times and among treatments. The level of significance was established at $p < 0.05$.

RESULTS

The postoperative course of healing was unremarkable. There were no adverse occurrences at either donor or recipient parietal bone sites.

Radiography and radiomorphometry

At 14 days, the percent radiopacity at the donor craniotomies in group 1 was significantly greater than

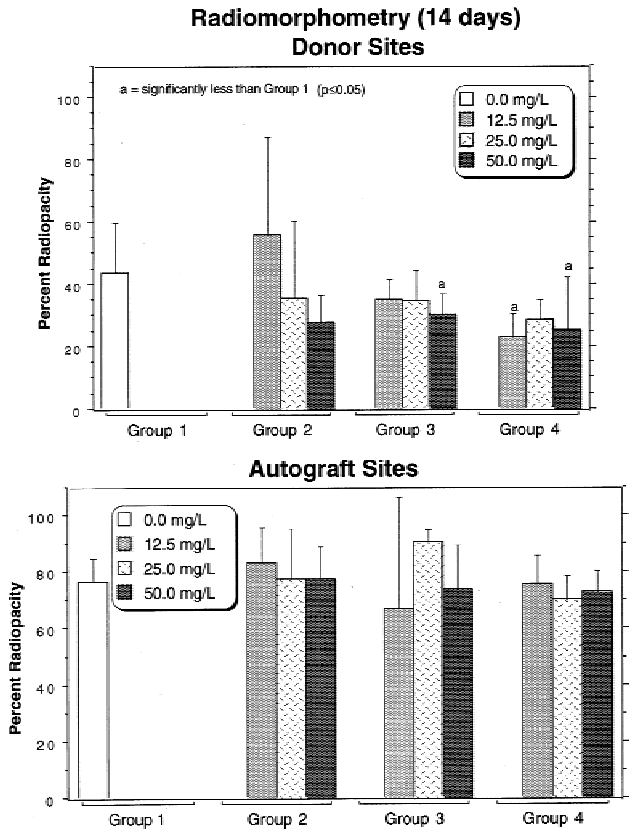

Figure 3. Histogram of radiomorphometric data at 14 days (mean + 1 standard deviation).

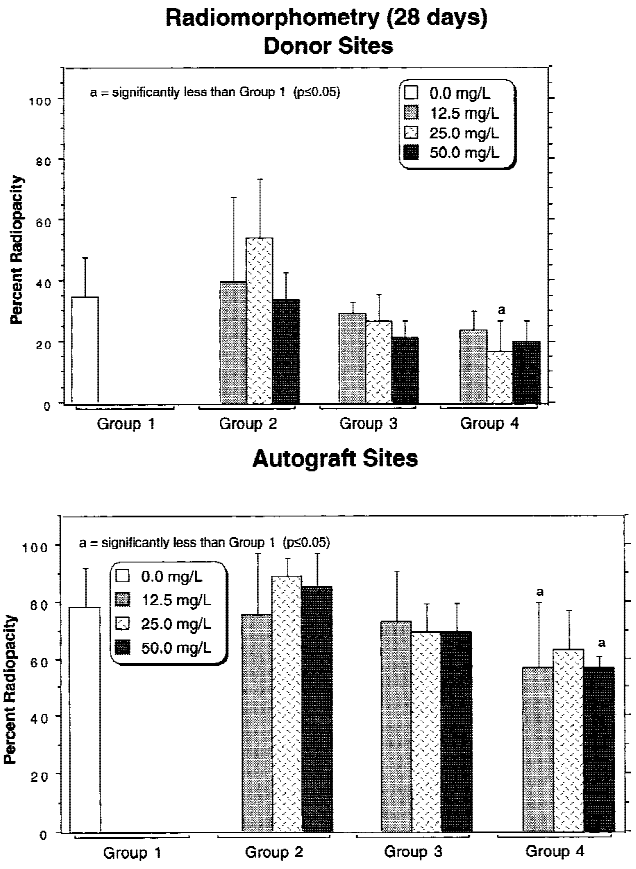

Figure 4. Histogram of radiomorphometric data at 28 days (mean + 1 standard deviation).

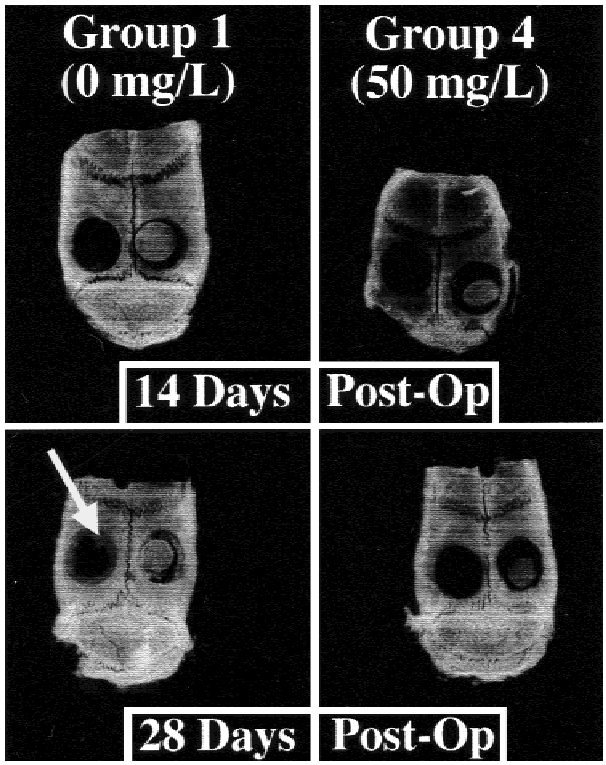

Figure 5. Typical radiographic response from either 0 or 50 mg/L of nicotine at 14 and 28 days. The spontaneously healing defects are to the reader's left and the radiopaque graft and recipient beds are to the reader's right. The white arrow indicates centripetally advancing new bone formation.

donor defects in group 3 receiving 50 mg/L nicotine and in group 4 receiving 12.5 and 50.0 mg/L nicotine (Fig. 3). There were no dose-dependent responses in the donor defects among the nicotine-treated groups. For the autograft sites, the amount of radiopacity was not dose dependent on nicotine (Fig. 3).

At 28 days the donor defects in rats given nicotine throughout the study exhibited a time-dependent decrease in radiopacity from 14 to 28 days. There was significantly less radiopacity in group 4, who were administered 50.0 mg/L, compared to other groups (Figs. 4, 5). For the autograft sites, the amount of radiopacity was not dose-dependent on nicotine for groups 1–3; however, data from group 4 cohorts (12.5 and 50.0 mg/L) suggested less new bone than group 1 (Fig. 4).

Histological and histomorphometry

At 14 days there was neither a difference in the histological appearance nor in the quantity of bone formation within the spontaneously healing donor sites (Figs. 6, 7). Minimal osteoblastic activity, limited new trabeculae, and fibrotic connective tissue were apparent. Osseous integration between the autograft and host bone at recipient sites was not evident; however, some new bone deposition could be observed with the lower nicotine doses. By 28 days the donor sites in group 1 had more new bone than group 2 (50.0 mg/L nicotine), group 3 (all nicotine doses), and group 4 (12.5 and 25.0 mg/L nicotine; Figs. 8, 9). Furthermore, some of the spontaneously healing donor sites for the 0 mg nicotine revealed osseous bridging (Fig. 8). Moreover, this same treatment group also had substantial osseous formation between the autograft and recipient bone (Figs. 8, 10). However, histomorphometric data did not indicate significant differences among treatments for the autografts, whereas dosing and timing differences did promote different bone healing responses in the spontaneously healing defects (Fig. 9). Specifically, the 0 dose of nicotine and the lowest duration of administration time supported the greatest amount of new bone formation.

DISCUSSION

We proved our hypothesis that in a characterized osseous wound model nicotine administration will hinder bone regeneration. Moreover, the timing and

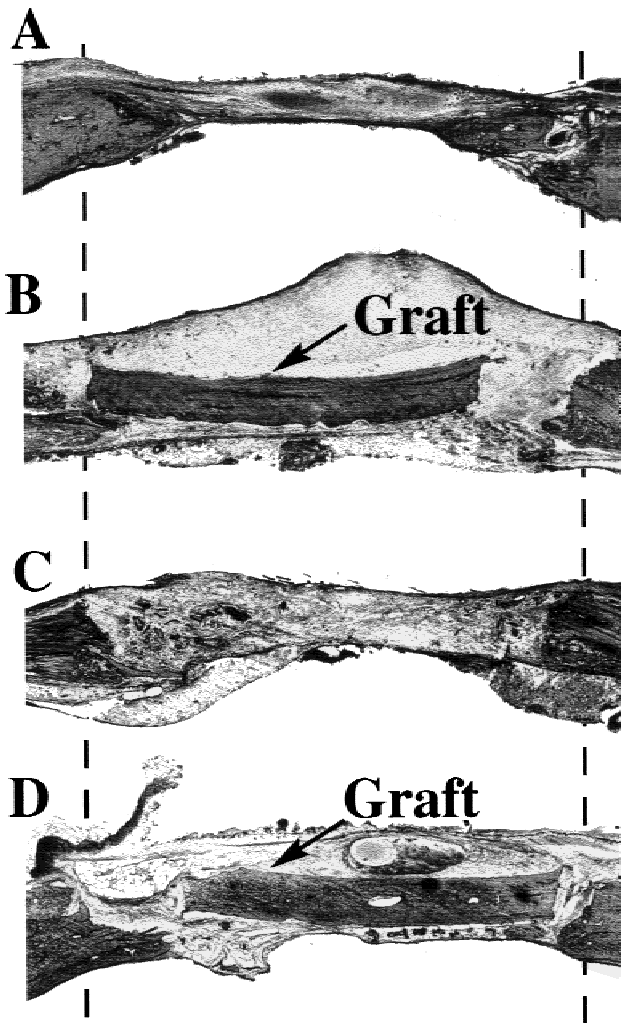

Figure 6. Representative coronal sections at 14 days of (A,C) donor and (B,D) recipient bone healing sites for 50 and 0 mg/L nicotine. Fibrous tissue prevails in donor defects and minimal new bone formation is apparent at autograft–host margins. Dashed lines indicate the wound edges. Autografts were inserted dural surface cephalically to preclude an exact fit; original magnification ×1.25; Goldner trichrome stain.

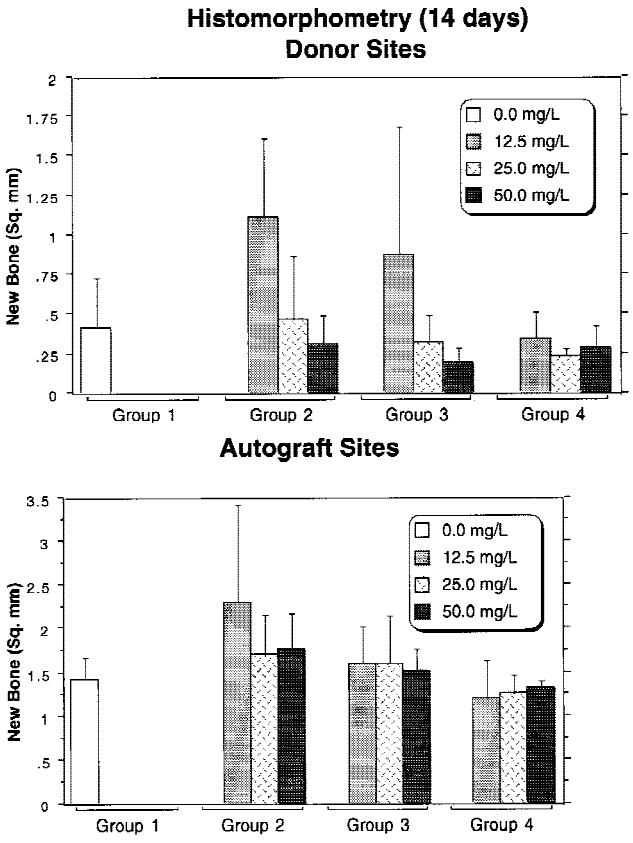

Figure 7. Histogram of histomorphometric data at 14 days (mean + 1 standard deviation).

sequence of nicotine administration adversely influenced bone healing. Furthermore, the negative impact from nicotine decreased bone healing more severely at 28 days than at 14 days. These findings underscore the importance for abstaining from nicotine-containing products prior to and following osseous procedures.

The data reported suggest early (14 days) osseous healing is affected less by nicotine than later healing (28 days). It is possible at the healing bone wound during the early phase that sufficient endogenous cells and signaling factors promote the healing cascade whether nicotine is present or absent. Review of the literature verifies the inherent capacity for bone to regenerate due to local cells and signaling molecules.[22] When osseous healing exhausts the localized supply of cells and signaling molecules, renewal is contingent upon vascularity and operational activity of endogenous cells activated during the early phase of bone repair. However, these events become subdued as a consequence of nicotine administration, which causes peripheral vasoconstriction at donor and recipient beds[13] and decreases osteoblast activity.[23–25] We further reason that peripheral vasoconstriction at the graft recipient bed reduces a potential osteoblast progenitor cell pool (i.e., the pericytes[26–28]) and produces an inhospitable environment jeopardizing bone repair potential and graft survival.

In the animal wound model we described, we were neither able to prove that different doses of nicotine had an effect on the healing process nor that nicotine deterred autograft incorporation. Possible reasons for these observations are variability in delivered nicotine doses from drinking water and the low bioavailability of swallowed nicotine (in the drinking water).[29] Orally administered nicotine undergoes a significant first-pass effect from hepatic metabolism (approximately 85–90%); whereas nicotine administration from other routes (e.g., from smoking) circumvents first-pass metabolism.[29] Therefore, to achieve nicotine plasma levels consistent with those likely from cigarettes (10–70 ng/mL[10,30]), our current experimentation with nicotine administration is being accomplished by nasal spray supplemented with a transdermal patch.[31,32]

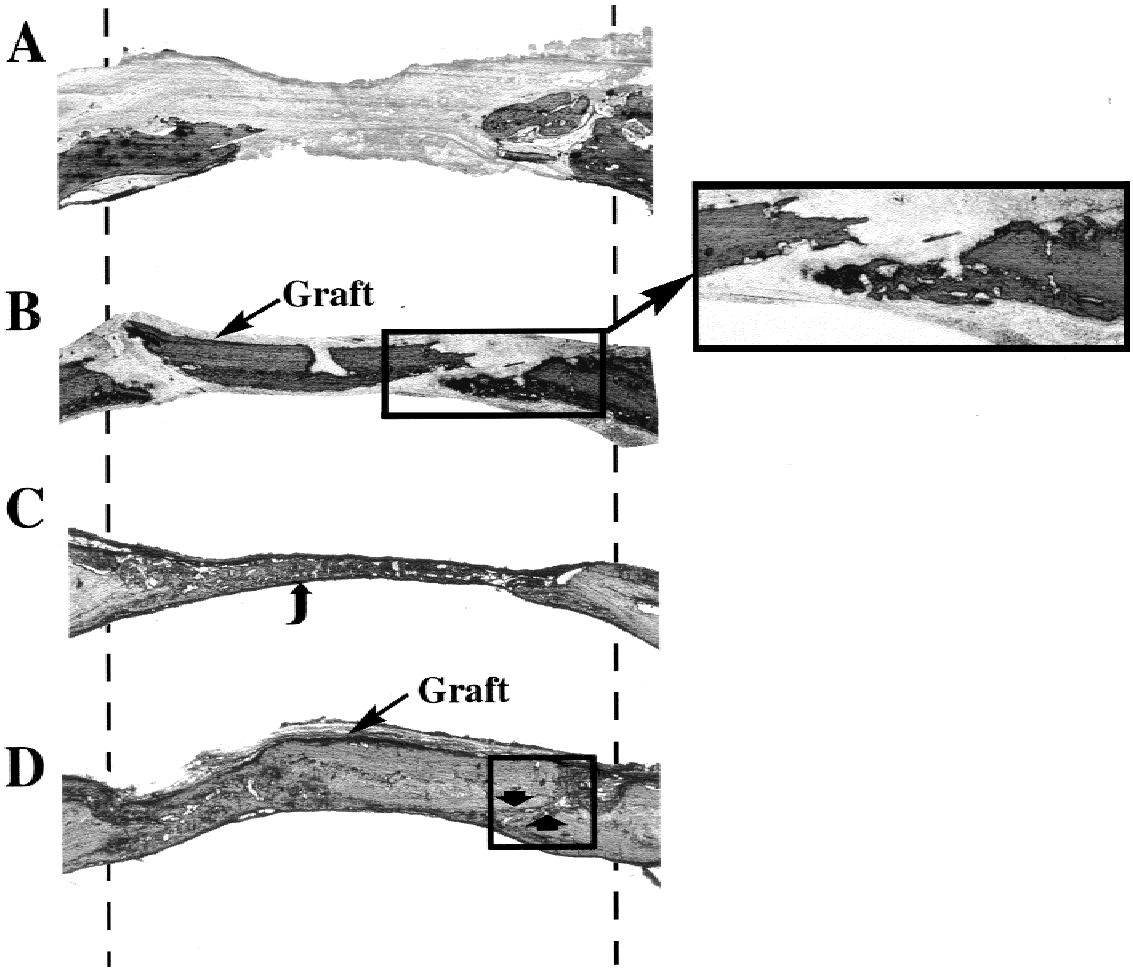

Figure 8. Representative coronal sections at 28 days of (A,C) donor and (B,D) recipient bone healing sites for 50 and 0 mg/L nicotine. (A) Fibrous tissue prevails in the 50 mg/L defect whereas (C) new bone formation (curved arrow) spans the 0 mg/L site. (B) The autograft appears to be undergoing degenerative changes. Irregularities at the graft–host interface and minimal bone formation reflect the negative impact of nicotine (insert). (D) In contrast, the autograft from the 0 mg/L dosing presents evidence of new bone proliferation at the graft–host interface (arrows). Dashed lines indicate the wound edges. Autografts were inserted dural surface cephalically to preclude an exact fit; original magnification ×1.25; Goldner trichrome stain.

These routes will also ensure a more reliable dosing regimen than nicotine in drinking water. Two contingencies can be considered if nasal and transdermal routes become unsuitable: either subcutaneous infusion by a miniosmotic pump (Alzet®, Palo Alto, CA) or smoking chambers where "research cigarette smoke" will be pumped into the chamber. The latter method is less favorable than the former due to the multiple agents in "smoke" versus a single agent (i.e., nicotine).

Another reason we could not detect differences in autograft incorporation among treatment groups could be related to the bone regenerating properties of the autogenous graft.[33] Moreover, the amount of interface area between the autograft and the host bone could have been sufficiently small to offset the negative impact that nicotine had on the healing wound. In contrast, a donor site with significantly less bone than the autograft recipient site could be more severely handicapped in the healing response during nicotine administration, despite the possibility of variable nicotine serum levels.

Studies in progress involve transdermal and nasal nicotine administration to prove the hypotheses that effects of nicotine on bone repair are dose dependent and that nicotine adversely impacts autograft incorporation. Furthermore, we are perfecting a rabbit model. The rabbit calvaria has an inner and outer table of Haversian and lamellar bone with intervening diploe, in contrast to the rat calvaria, which is predominantly thin cortical lamellar plates. Therefore, measuring autograft incorporation in the rabbit calvaria in response to nicotine will have more clinical relevance to humans than the described rat model.

Temporal sequencing of individual cellular and molecular events in the osseous wound sites were not determined in this study, although reports and reviews describe these phenomena in fracture re-

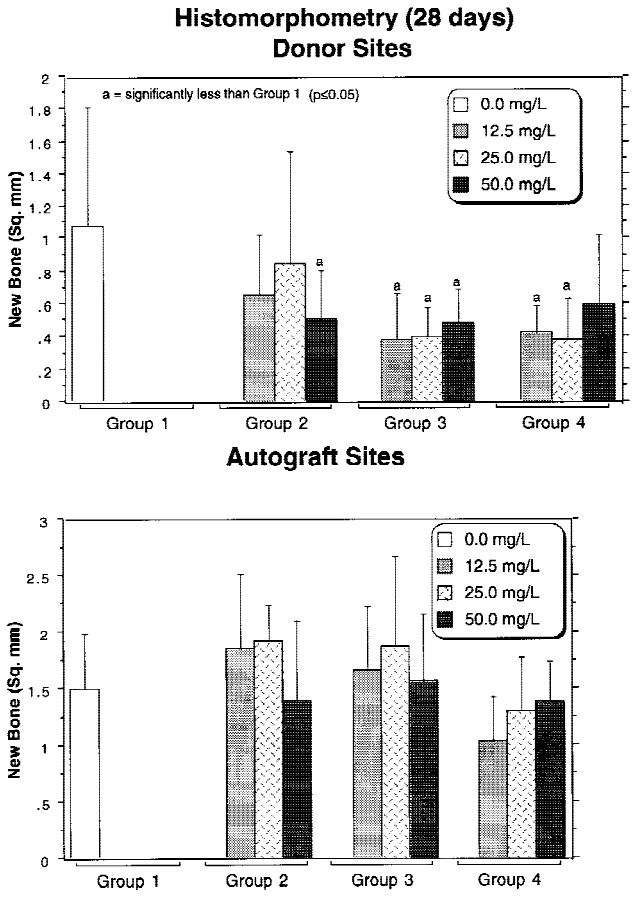

Figure 9. Histogram of histomorphometric data at 28 days (mean + 1 standard deviation).

pair.[22,34,35] We speculate a nicotine-induced decrement in signaling molecules and cells would be consistent with impaired bone healing. A rational therapeutic could be developed to offset this effect, as well as to promote peripheral vascularity. We offer this logic as the foundation to define and perfect a convenient bone wound model that could be exploited to develop clinical therapies.

In conclusion, we quantitated nicotine's impairment to bone repair. To the best of our knowledge, this is the first report on a clear and unambiguous method to measure bone formation at a spontaneously healing wound and an autograft recipient site to study the effects of nicotine. The lessons learned from this experience will enable our laboratory, as well as others, to improve the model, thus leading to the development of clinical remedies to reverse detrimental effects from tobacco products in patients who will require osseous procedures.

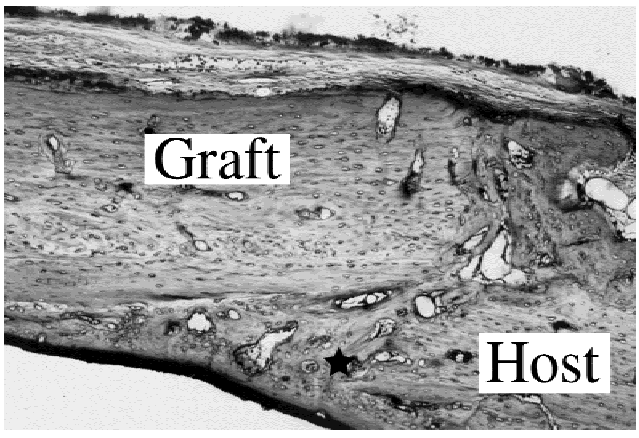

Figure 10. This higher magnification of Figure 8 (D) presents the *new bone formation between the graft and host; original magnification ×5; Goldner trichrome stain.

References

1. Thaller S. Immediate bone grafting in craniofacial trauma. In: Manson P, editor. Cranio-maxillofacial trauma. Philadelphia, PA: W.B. Saunders; 1991. p 408–419.
2. Brown CW, Orme TJ, Richardson HD. The rate of pseudoarthrosis (surgical nonunion) in patients who are smokers and patients who are nonsmokers: a comparison study. Spine 1986; 11:942–943.
3. Hanley EN, Levy JA. Surgical treatment of ischemic lumbosacral spondylolisthesis, analysis of variables influencing results. Spine 1989;14:48–50.
4. Silcox D, Daftari T, Boden S, Schimandle J, Hutton W, Whitesides T. The effect of nicotine on spine fusion. Spine 1995;20: 1549–1553.
5. Jones J, Triplett R. The relationship between cigarette smoking to impaired intraoral wound healing: a review of evidence and implications for patient care. J Oral Maxillofac Surg 1992;50: 237–239.
6. Sweet J, Butler D. The relationship of smoking to localized alveolitis. J Oral Surg 1979;37:732–736.
7. Blumenthal SL, Baker J, Dossett A, Selby DK. The role of anterior lumbar fusion for internal disc disruption. Spine 1988;13: 566–569.
8. Hadley M, Reddy S. Smoking and the human vertebral column: a review of the impact of cigarette use on vertebral bone metabolism and spine fusion. Neurosurgery 1997;41:116–124.
9. Gilman A, Rall T, Niles A, Taylor P, editors. Goodman and Gilman's the pharmacological basis of therapeutics. Elmsford, NY: Pergamon Press; 1990. p 180–181, 781.
10. Jensen J, Goodson W, Hopf H, Hunt TK. Cigarette smoking decreases tissue oxygen. Arch Surg 1991;126:1131–1134.
11. Rohn J, Ramp W. Nicotine inhibits bone growth *in ovo.* J Dent Res 1987;667:220–224.
12. Pocock N, Eisman J, Kelly P, Sambrook P, Yeates M. Effects of tobacco use on axial and appendicular bone mineral density. Bone 1989;10:329–331.
13. Daftari TK, Whitesides T, Heller J, Goodrich A, McCarey B, Hutton T. Nicotine on the revascularization of bone grafts: an experimental study in rabbits. Spine 1994;19:904–911.
14. Riebel G, Boden S, Whitesides T, Hutton W. The effect of nicotine on incorporation of cancellous bone graft in an animal model. Spine 1995;20:2198–2202.
15. Pietila K, Laakso I, Ahtee L. Chronic oral nicotine administration affects the circadium rhythm of dopamine and 5-hydroxytryptamine metabolism in the striata of mice. Naunyn-Schmied Arch Pharmacol 1995;353:110–115.
16. Havilcek LL, Crain RD. Practical statistics for the physical sci-

ences. Washington, DC: American Chemical Society; 1988. p 153–156.
17. Lieber RL. Experimental design and statistical analysis. In: Lieber RL, editor. Orthopaedic basic science. Columbus, OH: Port City Press; 1994. p 631–635.
18. Hollinger JO. Animal models for assessing bone repair with special emphasis on poly(alpha-hydroxy acid) delivery systems. In: Brighton CT, Friedlaender G, Lane JM, editors. Bone formation and repair. Rosemont, IL: American Academy of Orthopaedic Surgeons; 1994. p 341–354.
19. Hollinger JO, Buck D, Schmitz JP. Quantitative light microscopy. A powerful tool to assess bone. In: Hollinger JO, Seyfer A, editors. Bone repair and regeneration. Philadelphia, PA: W.B. Saunders; 1994. p 463–475.
20. Marden LJ, Hollinger JO, Chaudhari A, Turek T, Schaub R, Ron E. Recombinant bone morphogenetic protein-2 is superior to demineralized bone matrix in repairing craniotomies defects in rat. J Biomed Mater Res 1994;28:1127–1138.
21. Schmitt J, Buck D, Bennett S, Skalla W, Chistoforou C, Buechter D, Gruskin E, Hollinger J. Assessment of an experimental bone wax polymer plus TGF-β1 implanted into calvarial defects. J Biomed Mater Res 1998;41:584–592.
22. Hollinger JO, Wong MEK. The integrated processes of hard tissue regeneration with special emphasis on fracture healing. Oral Surg Oral Med Oral Pathol 1996;82:594–606.
23. Fang M, Frost P, Iida-Klein A, Hanhn T. Effects of nicotine on cellular function in UMR 106-01 osteoblast-like cells. Bone 1991;12:283–286.
24. Ramp W, Lenz L, Galvin R. Nicotine inhibits collagen synthesis and alkaline phosphatase activity, but stimulates DNA synthesis in osteoblast-like cells. Exp Biol Med 1991;197:36–43.
25. Payne J, Reinhardt R, Dyer J, Maze C, Dunning D. Nicotine effects on PGE2 and IL-1beta release by LPS-treated human monocytes. J Periodont Res 1996;31:99–104.
26. Owen M. Lineage of osteogenic cells and their relationship to the stromal system. In: Peck W, Bone and mineral research. Amsterdam: Elsevier Science; 1985. p 1–25.
27. Sims DE. The pericyte—a review. Tissue Cell 1986;18:153–174.
28. Brighton CT, Lorich DG, Kupcha R, Reilly TM, Jones AR, Woodbury RA. The pericyte as a possible osteoblast progenitor cell. Clin Orthop Rel Res 1992;275:287–299.
29. DeSimone E, Scott D. Nicotine and caffeine abuse. In: Young L, Koda–Kimble M, editors. Applied therapeutics: the clinical use of drugs. Vancouver, WA: Applied Therapeutics, Inc. 1995. p 1–5.
30. Benowitz N, Jacobs P. Daily intake of nicotine during smoking. Clin Pharmacol Ther 1984;4:499–504.
31. Henningfield J. Nicotine medications for smoking cessation. N Engl J Med 1995;333:1196–1203.
32. Ellenhorn M, Schonwald S, Ordog G, Wasserberger J, Ellenhorn's medical toxicology: diagnosis and treatment of human poisoning. Baltimore, MD: Williams & Wilkins; 1997. p 1110–1121.
33. Goldberg VM, Stevenson S. Natural history of autografts and allografts. Clin Orthop Rel Res 1987;225:7–16.
34. Bolander ME. Regulation of fracture repair by growth factors. Proc Soc Exp Biol Med 1992;200:165–170.
35. Pan WT, Einhorn TA. The biochemistry of fracture healing. Curr Orthop 1992;6:207–213.