Bone associated with implants in diabetic and insulin-treated rats

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Abstract
Objectives: Diabetes is an increasingly prevalent disease with oral health manifestations. While diabetes clearly has an effect on bone, its impact on the healing of bone associated with dental implants is not completely understood. The purpose of this study was to measure bone response to implants in uncontrolled and insulin-controlled diabetic rats.

Material and methods: One hundred and fifty-two rats were divided into control, diabetic, and insulin groups. Rats received streptozotocin (65 mg/kg) to induce diabetes; animals in the insulin group also received a subcutaneous slow-release insulin implant. Titanium alloy implants (1.5 × 8 mm) were placed in the proximal tibiae of animals. Implants were harvested at 2, 7, 14, and 24 days and examined histologically. Bone or bone-like tissue adjacent to implants was quantified as a percent. Data were compared using a two-way analysis of variance (ANOVA) with time and treatment as primary independent factors.

Results: Time and treatment were significant factors in predicting bone response to implants (P < 0.0001). Mean bone volume peaked at day 7 and decreased over time to day 24. Mean bone volume percent at 2, 7, 14, and 24 days (± SD) was 8.2 (± 8), 22.9 (± 8), 18.8 (± 10), and 14.9 (± 9), respectively. Mean total bone volume percent (adjusted for day) for control, diabetic, and insulin groups (± SD) was 12.4 (± 9), 22.6 (± 10), and 17 (± 7), respectively. Bone volume adjacent to implants in diabetic rats was significantly greater than controls (P < 0.05). Diabetic animals treated with insulin were not statistically different from controls.

Conclusions: Induction of diabetes with STZ is associated with increased bone response compared with controls. This response was mediated by treatment with insulin.

Clinical evidence suggests that implants can be successfully placed in diabetic patients [Balshi & Wolfinger 1999; Kapur et al. 1999; Olson et al. 2000; Hamada et al. 2001; Farzad et al. 2002; Peled et al. 2003]. However, the success of implants in these patients may be slightly diminished [Fiorellini et al. 2000; Morris et al. 2000]. One study found that duration of diabetes may impact clinical success rates (Olson et al. 2000). Diabetes has been shown to adversely affect the periodontium and oral health [Taylor et al. 2004], but the mechanism of interaction between diabetes and oral health interactions is complex and not fully understood.

As bone density and mineralization are adversely affected by diabetes [Goodman & Hori 1984; Devlin et al. 1996; Ward et al. 2001; Graves et al. 2004], it is reasonable to expect that the healing of endosseous implants could also be impacted. Animal studies that examine the effects of experimental diabetes on the healing of implants,
however, have been contradictory. One study reported an increase in bone volume associated with hydroxyapatite implants placed in diabetic rats [el Deeb et al. 1990]. Giglio et al. (2000) documents an increased bone response adjacent to lamellar titanium implants placed in rat tibiae after 14 days, but the histological presentation was similar to controls at 30 days. McCracken et al. (2000) support these conclusions, finding a significant increase in bone volume associated with implants in diabetic rat tibiae after healing 14 days.

Other studies have documented a decrease in bone volume associated with implants in diabetic animals. In a study of rats with alloxan-induced diabetes, diabetic animals demonstrated 50% less bone volume than controls. Animals treated with insulin appeared similar to controls [Siqueira et al. 2003]. One study found no difference in bone volume adjacent to implants in diabetic animals compared with controls [Nevins et al. 1998]. Still other studies conclude that experimental diabetes is associated with decreased bone volume in an animal model adjacent to hydroxyapatite implants [Takeshita et al. 1997, 1998].

The purpose of this study was to document the bone volume response adjacent to titanium alloy implants placed in diabetic and insulin-controlled diabetic rats over time.

Materials and methods

Animal preparation

Animals were maintained in an AAALAC-accredited [American Association for Accreditation of Laboratory Animal Care] facility; protocols were approved by the institutional animal use review board. For this study, 152 Sprague-Dawley rats were utilized. Rats weighed approximately 350 g. Food and water were provided ad libitum; the room was maintained at 23 °C with 12-h light/dark cycles.

Rats were divided into three groups: control, diabetic, and insulin. Animals in the diabetic group received a parenteral injection of streptozotocin [60 mg/kg] diluted in phosphate-buffered saline. Control animals received an injection of saline only. Rats in the insulin group received an injection of streptozotocin (60 mg/kg) and a subcutaneous insulin pellet [Lin-Plant; LinShin Canada Inc., Toronto, ON, Canada]. This 7 × 2 mm insulin implant was inserted through a small incision in the scruff of the neck with a heavy-gauge needle (supplied by the manufacturer). The implant releases insulin at a steady rate, rather than as a bolus associated with insulin injections. Due to the relatively high cost of the insulin implant, an unbalanced study design was used, with 60 animals in the diabetic group, 60 in the control group, and 32 in the insulin group, for N = 152.

Three days after inducing diabetes, 1.5 × 8 mm threaded titanium alloy implants [Crystal Manufacturing, Birmingham, AL, USA] were placed in the proximal tibia of the rats. These implants were utilized as machined; implants were treated in the following manner: 20 min ultrasonic cleaning, three times rinse in 100% acetone, 20 min in 20% nitric acid at room temperature, and two times rinse in deionized water. Each rat received one implant. Rats were anesthetized using volatile gas, and were weighed and tagged for identification; a narcotic analgesic was administered. After shaving, the surgical site was prepared with alternating scrubs of surgical skin cleanser and 70% ethanol. Rats were placed on a surgical heat source and draped to present a sterile field. A 1 cm incision was made below the knee through the periosteum. Tissue was reflected to expose the medial proximal portion of the tibia.

A pilot hole was drilled using a surgical handpiece and a No. 4 round bur. An oblique-transverse bicortical osteotomy was created using a 1.3 mm twist drill. All surgery was performed using copious irrigation. Primary closure of the muscle layer was achieved using resorbable sutures; skin was closed using surgical staples [Autoclip; Braintree Scientific, Braintree, MA, USA]. Animals were monitored until alert and mobile. Blood glucose was measured using an electronic glucose meter [AccuCheck Advantage; Roche Diagnostics, Indianapolis, IN, USA] at the time of surgery and at sacrifice. Diabetes was defined as a blood glucose level greater than 300 mg/dl at the time of surgery.

Implants were allowed to heal for 2, 7, 14, or 24 days. Rats were euthanized with carbon dioxide inhalation.

Histomorphometric analysis

Tibiae were retrieved and cleaned of soft tissue. Specimens were fixed in phosphate-buffered paraformaldehyde for 12 h and were then dehydrated in a series of ethanol solutions. These were cleared with xylene and infiltrated with polymethylmethacrylate resin for 7 days. Because of the metallic implant in the tibiae, sections were prepared using a cutting and grinding technique to produce one histological slide per specimen [Donath & Breuner 1982] using and Exakt cutting system [Exakt Technologies Inc., Oklahoma City, OK, USA]. Final sample thickness was approximately 60 μm, which was as thin as was possible for our lab to produce and still retain the metallic implant in the section. Samples were stained with toluidine blue.

Samples were analyzed using a histomorphometric quantifying system, consisting of a Nikon light microscope [Nikon Corp., Tokyo, Japan], a Sony 3-chip CCD videocamera [Sony Corporation of America, New York, NY, USA], a microcomputer and a video capture board [Scion Corporation, Frederick, MD, USA]. Images were analyzed and histomorphometric values were determined using NIH imaging software [NIH Image; National Institutes of Health, Bethesda, MD, USA]. Bone or bone-like tissue [blue-staining tissue with architecture similar to that of bone and containing osteocytes] within 1 mm of the implant surface within the medullary canal was quantified. This quantity, bone volume percent, was defined as the area occupied by bone (in pixels) divided by the total area of the region. Bone was identified by thresholding and digitizing techniques to minimize operator area. Three quantities were computed: proximal bone volume percent, distal bone volume percent, and total bone volume percent.

Data analysis

Data were analyzed using SAS [SAS v.9; SAS Institute, Cary, NC, USA]. Primary independent variables of interest were treatment [control, diabetic, insulin] and time [2, 7, 14, and 24 days]. Dependent variables were proximal, distal, and total bone volume percent. A two-way general linear model regression analysis was conducted to analyze these data. Where the global test indicated significance, groups...
were compared using Tukey’s post hoc analysis. Proximal bone volume percent and distal bone volume percent were compared using a paired t-test. Correlation between blood glucose levels and bone volume percent was measured using Pearson’s correlation analysis. Blood glucose levels were compared using analysis of variance (ANOVA) and Tukey’s post hoc analysis. Alpha for all tests was set at 0.05.

Results

Blood glucose levels confirmed the onset of diabetes in the diabetic group (Fig. 1). Three animals did not demonstrate increased blood sugar levels and were excluded from the trial. Two animals in the insulin group had excessively low blood sugar levels (less than 50 mg/dl) and were excluded from the study. It was assumed that these animals did not develop diabetes and the addition of insulin depressed their blood glucose levels below that of normal controls. Blood glucose measurements at time of sacrifice confirmed retention of diabetic symptoms in all diabetic animals.

Rats in the insulin group maintained blood sugar levels similar to controls at 2, 7, and 14 days. At 24 days, however, blood sugar levels in the insulin group were similar to levels observed in the diabetic group. The insulin implant manufacturer was contacted, and it was determined that the insulin implant did not last for the duration of the experiment; animals should have received an additional implant at approximately day 20. Therefore, animals in the insulin group at 24 days were excluded from the study. Blood sugar levels at the time of surgery are shown in Fig. 1. Onset of diabetic symptoms was further confirmed by significant weight loss in the diabetic group compared with control and insulin groups (Fig. 2).

Time after implant placement (2, 7, 14, 24 days) was a significant factor for total, proximal, and distal bone volumes (P < 0.001). However, this effect did not appear to be linear; mean bone volume peaked at day 7 and decreased through day 24. Mean bone volume percent at 2, 7, 14, and 24 days (± SD) was 8.2 (± 8), 22.9 (± 8), 18.8 (± 10), and 14.9 (± 9), respectively (Fig. 3). No significant interaction effects (day × group) were present in this model. Data was controlled for group and missing data.

Treatment effects in this model were also highly significant (P < 0.001). Mean total bone volume percent (adjusted for day) for control, diabetic, and insulin groups (± SD) was 12.4 (± 9), 22.6 (± 10), and 17 (± 7), respectively (Fig. 4). The diabetic bone response was significantly greater than control and insulin groups (P < 0.05), which were not significantly different.

In general, more bone was present on the proximal side of the implant than on the distal (P < 0.001), as displayed in Fig. 5. This observation appeared to be especially prominent for animals in the diabetic group. This finding was consistent in virtually every specimen.

A weak but statistically significant positive correlation was noted relating blood glucose levels to bone volume associated with implants, with r = 0.48 (P < 0.001).

Histological presentation

Control: At day 2, very little blue-staining tissue was noted at the implant interface or in the nearby medullary canal. Where bone or osteoid was present, it was small and focally located. At day 7, the bone response was much more pronounced. Tissue was loosely organized and haphazard in nature. In some places, the tissue appeared to

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**Fig. 1.** Blood glucose levels at time of surgery (± SD). Diabetic animals demonstrated significantly greater blood glucose levels compared to controls and insulin groups (P < 0.001). Insulin and control groups were not significantly different.

**Fig. 2.** Weight of animals in grams at time of surgery (± SD). Diabetic animals demonstrated significant weight loss compared with control and insulin groups (P < 0.001).

**Fig. 3.** Bone volume percent by time (± SD). Mean bone volume percent peaked at day 7, and was lowest at day 2. Groups connected by a horizontal line are not significantly different; other values represent significant differences (P < 0.03).

**Fig. 4.** Mean bone volume by group (adjusted by day). Diabetic animals demonstrated significantly more bone associated with implants compared with control and insulin groups, which were not significantly different.

**Fig. 5.** Bone volume by location (proximal, solid; distal, hashed box). A paired t-test revealed a significant difference between proximal and distal bone percent values.
touch the implant surface. The volume of bone or osteoid tissue was much greater than at other time frames, and the quality of the tissue was similar to that found in diabetic specimens. After healing 14 days, bone was more organized in nature. It frequently approximated the surface of the implant. The bone appeared to be more laminar in nature and became aligned with the direction of the implant in the vicinity of the interface. At 24 days, bone was largely absent in the medullary canal but was lining most of the surface of the implant. This supporting bone was uniform in consistency and appearance and in adaptation to the implant surface. Images of specimens are presented in Fig. 6.

Diabetic: At day 2, slight amounts of bone or osteoid matrix was present in these specimens. Where present, this tissue presented as fine focal points of blue-staining tissue. The histological presentation was similar to controls at day 2, with more bone appearing in some samples. At day 7, bone volume had increased in exuberance around the implant site. Bone was loosely organized and of a woven nature. This was also similar to controls at the same time observation. At day 14, however, the bone remained pronounced in volume. Little change was noted in the quality of the bone from days 7 to 14 in diabetic animals through day 14 and into day 24. This may suggest compromised healing or remodeling occurs in these animals (toluidine blue).

In this study, we observed that when blood sugar levels were controlled by insulin, less bone was associated with the implants. This would support the theory that high glucose levels may lead to AGE formation and impact bone response. However, it is also noted that mean bone response in the insulin group was consistently higher than controls, even if the difference was not significant. This suggests that another mechanism besides high glucose levels may contribute to the increased bone formation. Such mechanisms could even be unrelated to diabetes, but an unexpected effect unique to STZ-induced diabetes. This could be the reason that Siqueira et al. (2003) found less bone associated with implants in Alloxan-induced diabetic rats.

Discussion

Glucose blood levels and weight changes in the diabetic group confirmed onset of diabetic symptoms (Wong & Tzeng 1993; Wong & Wu 1994). The diabetic animals had significantly higher blood glucose levels and increased weight loss. The diabetic animals controlled by insulin were not significantly different than controls for these parameters.

Contrary to intuition, animals with STZ-induced diabetes produced more bone adjacent to implants. One possibility for this observation could be glycosylation of proteins in diabetic animals. Reaction of protein lysyl residues with sugars, glucose for example, results in formation of a Schiff base which can rearrange to form an Amadori product. Subsequent irreversible oxidation, rearrangement and fragmentation reactions result in advanced glycation end product (AGE) formation. AGE modification of long-lived connective tissue proteins is thought to underlie many of the pathological conditions associated with diabetes and, over a longer time frame, with normal aging (Khalifah et al. 1999; Cooper 2004).

In this study, we observed that when blood sugar levels were controlled by insulin, less bone was associated with the implants. This would support the theory that high glucose levels may lead to AGE formation and impact bone response. However, it is also noted that mean bone response in the insulin group was consistently higher than controls, even if the difference was not significant. This suggests that another mechanism besides high glucose levels may contribute to the increased bone formation. Such mechanisms could even be unrelated to diabetes, but an unexpected effect unique to STZ-induced diabetes. This could be the reason that Siqueira et al. (2003) found less bone associated with implants in Alloxan-induced diabetic rats.

Giglio et al. (2000) report a similar bone response to implants in diabetic rats over
time as is reported here. These authors suggest that the response may be due to a lack of bone remodeling in the case of the diabetic animal. They note that the histological appearance of the bone is more woven in nature, rather than lamellar. They conclude that the healing process is slower in STZ-induced diabetic animals. The histological appearance of the bone in that study reflects the data reported here; however, we add observations at 2 and 7 days. It is possible that diabetes-related healing abnormalities may be causing a delay in the remodeling and maturation of the bone adjacent to implants. In this case, it would be concluded that the diabetic animals have more bone volume associated with implants because healing is compromised; for example if osteoclast activity is inhibited.

Of interest was an observation that diabetic animals may produce ‘bone’ or osteoid-type matrix more quickly than controls. Stratified by day, at day 2 diabetic animals have a 15% bone volume adjacent to implants, while controls have less than 3%. This difference at only 2 days after implantation is not likely due only to remodeling factors. It appears that diabetic animals produce bone more quickly than controls. Animals treated with insulin were again in the middle of these groups, with 10% bone response, suggesting that insulin also mediates bone production in the early stages of healing.

An increased response on the proximal portion of the implant has been previously noted [McCracken et al. 2000] and was also demonstrated in these data. This observation appears to be especially prominent in the diabetic group. This could be a function of greater blood supply on the proximal portion of the implant, closer proximity to the growth plate and a richer source of mesenchymal stem cells, a result of occluding the medullary canal with the implant and hindering cell recruitment, mechanical considerations, or a combination of these factors. Regardless of diabetic status, these data suggest that bone growth adjacent to implants is influenced by anatomic position as well as other host and material factors. Regarding clinical application of these data, it is recognized that STZ-induced diabetes in a small animal model may or may not have a direct correlation with the human diabetic clinical situation. However, these data will hopefully add to our knowledge base regarding host, implant, and diabetic interactions.

From this study, it is concluded that time and treatment factors are significant when predicting bone response to implants in normal and diabetic rats. Bone response peaks around day 7 in these animals. Diabetic animals produce more bone adjacent to implants than controls. This response is mediated by insulin treatment.

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References


