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Authors
VanSchoiack, Lindsey R
Shubayev, Veronica I
Myers, Robert R
et al.

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In Vivo Evaluation of Quantitative Percussion Diagnostics for Determining Implant Stability

Lindsey R. VanSchoiack, PhD[1], **Veronica I. Shubayev, MD**[2,3], **Robert R. Myers, PhD**[2,3], **Cherilyn G. Sheets, DDS**[4], and **James C. Earthman, PhD**[1,5]

[1]Dept. of Biomedical Engineering, University of California, Irvine, CA 92697-2715

[2]Dept. of Anesthesiology, University of California, San Diego, La Jolla, CA 92093-0629

[3]Veterans Administration San Diego Healthcare System, La Jolla, CA 92161

[4]Newport Coast Oral-Facial Institute, Suite 200, 360 San Miguel, Newport Beach, CA 92660

[5]Dept. of Chemical Engineering and Materials Science, University of California, Irvine, CA 92697-2575

Abstract

Purpose—A percussion instrument (Periometer®, Perimetrics LLC, Newport Beach, CA, USA) and rat model were used to test the hypothesis: percussion diagnostics provides reliable, reproducible indications of osseointegration.

Materials and Methods—Titanium implants were placed in femurs of 36 Sprague-Dawley rats. Each animal was assigned to one of six groups of six defined by one of three time points (2, 4, or 8 weeks post-placement) and one of two treatments (MMP inhibitor or vehicle). Percussion testing was conducted three times/subject at implant placement and at one of the time points. For each time point, there was an experimental group that received daily intraperitoneal injections of GM6001, and a control group that received no MMP inhibitor. The percussion data consisted of loss coefficient (LC) values that characterize energy dissipation. Statistical analysis was performed on the LC values for two animal groups using the paired Student *t* test to assess differences as a function of time, and the independent *t* test to compare mean LC for the study groups at sacrifice (α=0.05). Histological evaluation using the osteogenic CD40 protein marker was also performed.

Results—A nearly significant difference in mean LC at the 2-week time point was observed between the two treatments with the GM6001 group having the higher value (p = 0.053). There was a greater difference between the mean LC values for the 4-week GM6001 and vehicle groups (p = 0.001). The histological evidence for subjects in these two groups confirmed reduction of osteogenesis at the implant interface after administration of the MMP inhibitor.

Conclusions—Lower vehicle LC values relative to the GM6001 therapeutic group were observed, consistent with the effect MMP inhibition has on matrix remodeling at the implant bone

Corresponding author: James C. Earthman, PhD., Depts. of Chemical Engineering & Materials Science and Biomedical Engineering, ET 916, University of California, Irvine, CA 92697-2575. VOICE: (949) 824-5018, FAX: (949) 824-2541, earthman@uci.edu.

interface. This finding in conjunction with histological observations confirms that osseointegration can be monitored using percussion diagnostics.

Keywords

implant; matrix metalloproteinase; osseointegration; *in vivo* quantitative percussion diagnostics

INTRODUCTION

Successful implants must meet long-term mechanical and esthetic needs of patients. An instrument that could provide lifetime quantifiable measurements of implant stability and surrounding bone quality would be an advantage to patients and the dental industry.[1] Current methods used to measure bone quality and stability at implant sites have limitations. Radiography is difficult to standardize for position and representative of only two dimensions, while dual-energy X-ray absorptiometry (DXA) scans are cost prohibitive, radiation intensive and time consuming.[2] The conventional practice of tapping the implant with a metal instrument to make an auditory assessment is not quantitative. Meanwhile, removal torque is problematic for implants in cancellous bone and can precipitate failure in minimally osseointegrated implants.[3] Resonance frequency evaluations are useful, but have limitations related to the need for disassembly and implant geometries.[4] However, it is important to track the stability of implants during healing and loading since even small changes in bone density and structure can significantly affect stability.[5]

Osseointegration is the “continuing structural and functional coexistence” of an implant and the bone in which it is placed to provide a stable interface to transmit loads without invoking a large immune response.[5–7] Similar to the natural tooth complex, an implant and its supporting bone exhibit a combination of elastic and anelastic (time-dependent) behaviors. If the implant and supporting bone were to behave with a strictly elastic response, the loss coefficient (LC) would be zero because no energy would be dissipated.[8,9] However, restorative materials and bone are not strictly elastic and therefore provide some energy dissipation during loading so that LC > 0. If the bone becomes damaged or does not properly osseointegrate, additional energy dissipation can occur due to excessive frictional micro-motion at defects within the bone or at the bone-implant interface. Thus, it follows that a reduction in osseointegration should result in an increase in loss coefficient for a given implant.

Immediate loading protocols have an implant surgically placed in the jawbone, which is restored with an immediate provisional restoration allowing transmission of some level of occlusal forces to the bone. Two-stage implants are surgically placed and submerged to allow for bone healing and osseointegration before they are fitted with a provisional restoration. Despite the advantages of fewer surgeries, a quicker return to a normal diet, and possible improved esthetic outcomes, immediate loading has some potential drawbacks. Delayed loading has a success rate of about 96% at endpoints more than a year out, while immediate loading has been reported by some authors to have a lower (~ 80%) success rate at 12 to 18 months after placement for randomly selected patients, including those who were parafunctional or had implants placed in extraction sites.[1,10–12] Improvements in protocols

may continue to increase overall success rates, but without a reliable method for periodically monitoring the level of osseointegration an increased risk of failure may persist for immediate loading protocols.[11,13]

Bone matrix turnover is regulated by the extracellular zinc-endopeptidase family of matrix metalloproteinases (MMPs), which includes collagenases, gelatinases, matrilysins, stromelysins and membrane-type MMPs.[14] It has been shown that MMPs are important to the formation of properly constituted extracellular matrix (ECM) during integration of pure titanium threads with newly forming bone.[15–17] Accordingly, the inhibition of MMPs should lead to less osseointegration over a given period of time after an implant is placed in bone.

Instrumented percussion measurements of the loss coefficient were used in the present study to identify the quality of bone, the initial stability, and the osseointegration level of implants over their functional life. The working hypothesis is: loss coefficient data obtained using percussion diagnostics will provide a reliable, reproducible indication of osseointegration as a function of time *in vivo*. To test this hypothesis, an established rodent femur model of osseointegration involving commercially pure titanium implants was used. The rate of osseointegration and the quality of implant stability over a series of time points were determined from instrumented percussion readings. The results were judged using a synthetic smallmolecule MMP inhibitor, GM6001, to interfere with the endogenous determinants of successful osseointegration *in vivo*. Rat femurs were implanted with threaded Ti implants perpendicular to the surface of the bone, just below the hip and exposed to one of two experimental conditions: no MMP inhibitors or MMP inhibitors. Osseointegration level was quantified in rat femurs implanted with Ti screws after periods of varying MMP activity. Percussion testing was used as a direct mechanical indication of the effect MMPs have on osseointegration. Histological evaluation was also perfomed for one time point to confirm the reduction in osteogenesis produced by the MMP inhibitor.

MATERIALS AND METHODS

Titanium Fixtures

Commercially pure (CP) titanium implants (Integrum AB, Göteborg, Sweden), were chosen for their compatible geometry. The implant screw was Grade 4 titanium with a 0.4 mm-long, smooth middle section, a 2 mm diameter by 2 mm-long M2 thread at the mesial end, and a 1.6 mm diameter by 1.6 mm long M1.6 thread at the distal end. A 0.4mm-deep slot was machined at the distal end of the implant to facilitate reverse torque removal. A CP Ti abutment was also provided with the implants. The abutment was used for both surgical placement and percussion testing. Ethanol sterilized fixtures were kept in dry glass containers and handled with titanium instruments to avoid contamination. All surgical implements were commercially pure titanium and a Dremel® Moto-Tool Model 395 (Robert Bosch Tool Corporation Racine, WI, USA) was used to hold the surgical burs.

Animals and Surgery

Female adult Sprague-Dawley rats (225–250 g, Harlan Labs Indianapolis, IN, USA), were used for the animal model. Thirty-six animals were equally divided into groups of six and

were assigned into one of six treatment modalities determined by treatment time (two, four and eight weeks) and drug treatment (GM6001 and vehicle). A previously conducted power analysis showed that statistical significance could be verified with four animals, and each group consisted of at least 4 rats for valid data sets. Animals were housed at 22°C under a 12 h light/dark cycle with *ad libitum* access to food and water. The animals were exposed to 4% Isofluorane (Baxter, Deerfield, IL, USA) at 1 L/min airflow by inhalation for the entire procedure. The site preparation was performed with a 1.7 mm diameter hand drill and threaded with an M2 pretapping device to a depth of 2 mm into the widest part of the femur. The abutment was threaded onto the distal end of an implant to facilitate placement into the surgical site. Each implant was placed to a depth of 2 mm and the animal was removed for percussion testing. Once this testing was completed the animal was taken back to the surgical field and the abutment was removed while holding the implant in place with a surgical screwdriver down the hollow throat of the abutment. The wound was closed with 3-0 absorbable sutures using continuous stitches. The animals were allowed to recover in a sensory-enriched environment without restricting their mobility.

Intraoperative percussion testing was performed, as detailed below, at 2, 4 and 8 weeks after implantation. All procedures conformed to NIH Guidelines for the Care and Use of Laboratory Animals and protocols approved by the Institutional Animal Care and Use Committee and the VA San Diego Healthcare System.

Quantitative Percussion Testing

The Periometer percussion instrument was used to measure the loss coefficient of the femur implants. Loss coefficient measurements determined by this medical device were shown to correlate with simulated bone densities in a previous in vitro study.[18] The system is composed of a hand piece and control unit, power supply, and custom computer software to control the testing, acquire data, and provide analysis and visualization of the results. Instrument calibration was preformed prior to each use to assure data precision. Calibration is accomplished by testing two material standards with known loss coefficient values (Al alloy 6061 and polytetrafluoroethylene).

Percussion testing was conducted twice on each animal. The first test was performed immediately following implant placement and before suturing soft tissue over the implant (Figure 3). Immediately following the first test, each animal returned to the surgical field for incision closure. The second test was conducted at the time of sacrifice. For each test, three percussion measurements were performed in succession providing a total of 30 percussions, 10 for each measurement. Reproducible accuracy of the data was assured by the requirement that the standard deviation in the loss coefficient had to be below 0.002 (less than 2% of the LC value). In the rare instance that this requirement was not met, the percussion test was immediately performed again.

MMP Inhibitor Therapy

A specific, broad-spectrum small-molecule MMP inhibitor GM6001 (Calbiochem, Novabiochem International, Inc, La Jolla, CA, USA) in vehicle (ethanol in filter sterilized buffered saline) were prepared according to the manufacturer's instructions and

administered at 60 μg/rat/day by intraperitoneal injection. These injections started the day after surgery and were continued until the day before sacrifice. Six animals were in each experimental group with Ti implants and MMP inhibitor, and six animals were in each vehicle group with Ti implants and no MMP inhibition.

Tissue Isolation and Immunohistochemistry

The animal subjects were anesthetized at the desired time point after implant placement. Any periosteum that had formed around the top of the implant was removed and the testing abutment was attached for the second set of percussion tests. The animals were perfused transcardially with fresh 4% paraformaldehyde (PFA) in 0.2 M phosphate buffer. The femurs were then resected, cleaned and post-fixed in 4% PFA solution at 4°C for 48 hours. The bones were rinsed in a phosphate buffer and in deionized water. The vials were filled with Immunocal® (Decal Chemical Corporation, Tallman, NY, USA) for decalcification and the bones were cut distal and proximal to each implant.

The implant/bone samples were embedded in paraffin and cut into 10 μm sections. The sections were then put into a solution of 1% Sta-On® Tissue Selection Adhesive (Surgipath, Richmond, IL, USA) in a 50°C flotation bath, mounted onto slides and dried on a slide warmer at 60°C for one hour. The slides were then baked overnight at 37°C prior to staining. Immunohistochemical staining was performed for CD40 protein, which promotes bone formation, calcification, and osteoclast genesis.[19] Thus, the expression levels of this protein should be higher in growing bone and lower when bone growth is suppressed by factors such as the inhibition of MMPs. The sections were deparaffinized with xylene and rehydrated in a series of graded ethanol ranging from 100 to 70%, followed by phosphate-buffered saline (0.01 M PBS, pH = 7.4).

Endogenous peroxidase was blocked with 3% hydrogen peroxide, followed by DAKO antigen retrieval (DAKO, Carpinteria, CA, USA) application for 300 s at 95°C. Nonspecific binding was blocked with 10% horse serum for an hour at room temperature. The sections were incubated overnight with anti-CD40 antibody (Abcam, San Francisco, CA, USA). The sections were then rinsed in PBS, followed by the application of biotinylated goat anti-rabbit (Vector, Burlingame, CA, USA) for an hour at room temperature. The avidin-biotin complex (Vector) was applied for an hour at room temperature. After rinsing with PBS, the sections were developed with 3’3-diaminobenzidine (Vector), counterstained with methyl green (Fisher), dehydrated, and mounted with Entellan medium (Merck, Darmstadt, Germany). Control sections with nonimmune serum from the rabbit (or mouse) animal source were used as control. The imaging was performed using a Leica DMRB microscope (Leica Microsystems, Buffalo Grove, IL, USA), a Leica DFC 300 camera, a desktop computer, and Openlab 3.1.2 image analysis software (PerkinElmer, Waltham, MA, USA).

Percussion Data Analysis

The loss coefficient (LC) was used to characterize the energy dissipation response of the implant and surrounding bone to mild percussion. As implied in the hypothesis, energy dissipation indicated by the LC was expected to decrease as osseointegration progressed. The percussion response, characterized by energy return vs. time, was checked for

irregularities that can indicate defects in the supporting bone.[18] For each experimental condition within each sacrificial time group, paired Student *t* test was used to assess the change in LC means between implant placement (baseline) and sacrifice time. For each sacrifice time, the difference between the experimental LC mean and control vehicle mean was assessed using an independent *t* test. In Case 1, vehicle sacrifice data versus vehicle implant placement data were analyzed. In Case 2, GM6001 sacrifice data versus GM6001 placement data were compared, and in Case 3, vehicle sacrifice data versus GM6001 sacrifice data were evaluated.

RESULTS

Percussion Testing

Energy return data for typical percussion tests were plotted for each group and individual animal. As represented by the data in Figure 2, none of the energy return peaks were overtly skewed nor contained additional peaks that can be indicative of defects in the support structure, implant movement, or loose test abutments.[18] Rather, uniform bell-shaped energy return peaks indicated that all the implants were securely implanted the day of surgery. The implants were in contact with the medullary channel at their distal ends, but the support of the system came from the cortical and cancellous bone in contact with the implant along the majority of its length.

The resulting LC data were analyzed to identify differences between the values of inter and intra time point groups. Figure 3 shows individual LC values, each based on 30 percussions, as a function of weeks after implant placement for both vehicle and GM6001 treated subjects. Each experimental group data set was analyzed to determine if the LC values were normally distributed. Also, we used the quartile method to exclude data that were atypical for three animals. In the 2-week group, one GM6001 data point was excluded for being a low outlier according to this method. In the 8-week data sets, two vehicle data points were excluded, one due to an animal death and another due to an unusually high outlier according to the quartile method. None of the experimental groups showed observable side effects from the daily injections. The animal subjects remained active and docile for the entire study.

Morbidity was limited to four animals. One animal had a nerve irritation from the surgery that caused dragging of the left hind foot, but full dexterity returned by the time of sacrifice. Another animal formed a subdermal pustule slightly distal to the implant that did not affect the mobility or energy level of the animal. Finally, two animals experienced lethargy after implant placement, which responded to antibiotic therapy.

Mean LC values for the different week of sacrifice groups are plotted in Figure 4 and the corresponding statistical findings are listed in Table 1. The results indicated measurable differences after just two weeks following implant placement. Specifically, a nearly significant difference ($p = 0.053$) is indicated in Table 1 between the GM6001 and vehicle group means where the former group exhibited the higher value (Figure 4). However, there was no significant change in LC mean from the baseline values for either treatment group for this time point.

The mean LC values for the 4-week sacrifice groups were significantly different from their starting baseline values and from each other as indicated in Table 1. Therefore, the null hypothesis that the mean LC values were the same was rejected for all three cases. The data for both groups at this time point had no significant outliers as defined by the quartile criterion. Both the *p* values in Table 1 and the plotted data in Figure 4 demonstrate clear distinction between the two treatment groups at each time point. In addition, both groups exhibited lower mean LC values than their corresponding initial values, as expected for the osteoblastic phase of bone remodeling. We note that all of the LC values were lower for the vehicle group in Figure 3 at the 4 week time point. Accordingly, the significantly lower vehicle LC means listed in Table 1 relative to the GM6001 therapeutic group indicate that this MMP inhibitor was still effective in altering the matrix remodeling at the implant-bone interface. Further, the overall decrease in LC values for both groups also shown in Table 1 indicates bone growth molecular activity was occurring in all subjects at this time point. Thus, the results for 4 weeks clearly support the hypothesis that percussion diagnostics provides reliable and reproducible indications of osseointegration.

The mean LC values after eight weeks from implant placement returned to near initial values as indicated in Table 1 and Figure 4. This implied decrease in implant stability for both groups suggests that a large number of osteoblasts have become osteocytes by this time point and are maintaining and remodeling as opposed to primarily depositing bone. Accordingly, we hypothesize that the bone at eight weeks of healing in both groups is primarily undergoing stabilization instead of building activity. Also at this time point, the mean LC value for the GM6001 group was still significantly greater than that for the vehicle group ($p = 0.026$). Thus, it appears that the MMP inhibitor is still effective at this time point in altering the bone remodeling process for the present animal model.

CD40 Levels at the Implant-Bone Interface

The histological evaluation and reactivity for the osteogenic CD40[19], in bone slices neighboring the implants indicated successful titanium osseointegration at four weeks after implantation of the vehicle treated bones (Figure 5). While both Figure 5a and 5b show that CD40 is present in the nuclei of cells, the locations of these cells are strikingly different. The vehicle group exhibited a large number of CD40 containing nuclei at the interface of the bone and implant. By contrast, the CD40 protein is not observed directly along the implant/bone interface in the GM6001 treated bone. The staining levels were the greatest at four weeks after implant placement, increasing from those for the two-week group and then were lower at eight weeks after implant placement (data not shown). To confirm this finding quantitatively, binary images were produced using image analysis software so that the stained nuclei were differentiated from the rest of the bone tissue. Area analysis of the stained nuclei in Figure 5 indicated that the CD40 containing cells corresponded to approximately 10% of the area of the vehicle bone while they constituted only 2.3% of the GM6001 treated bone area. Thus, the present histological results are clearly consistent with the percussion results for the 4 week time point in Table 1 indicating that greater osseointegration occurred for the vehicle animals than for the GM6001 administered group.

DISCUSSION

It appears that the initial increase in implant stability (lower LC) for the vehicle group was due to a natural rapid rate of bone turnover in the rodent model. The large ratio of implant contact surface area to total bone at the implant site may also have contributed. By contrast, the mean LC value is still relatively high after two weeks, apparently as a result of initial resorption prior to bone growth, when the natural dissolution process is therapeutically affected by GM6001. This finding is consistent with several reports, which indicate that the inhibition of MMPs breaks the signaling process that controls the switch from osteoclastic to osteoblastic cellular activity.[20–23] The present work suggests that loss coefficient measurement using percussion diagnostics is an alternative to other methods used for *in vivo* tracking of osseointegration levels.[7,24,25] The value of LC changes due to natural and modified healing, as well as physiological changes in the osseointegration level of the Ti implants.

The inhibition effect of MMPs was expected to slow the rate of implant osseointegration resulting in lower levels of stability.[15] The hypothesis that LC measurements could detect the altered speed of osseointegration due to the presence of MMP inhibitors was confirmed. The daily MMP inhibitor injections decreased implant stability, as consistently indicated by higher LC means (Figure 4), resulting in a significant difference between the data for the two experimental groups at the four week time point (Table 1).

Earlier studies have shown a trend of decreased stability immediately after implant placement followed by a period of significantly increased stability as the Osseointegration process continues.[7,24,26] The vehicle group data did not indicate decreased stability even at two weeks after implant placement. However, it is possible that decreasing stability occurred before the two-week time point due to a relatively high healing rate for rats. Rats undergo this process faster than the 18 to 24 months that it can take in humans.[27]

CONCLUSIONS

The results of the present study support the hypotheses that values of the loss coefficient determined by percussion diagnostics provide a reliable, reproducible indication of osseointegration as a function of time *in vivo*. Additionally, the use of a synthetic MMP inhibitor can be used to slow the osseointegration process. As noted in the data, the significantly lower vehicle LC mean relative to that for the GM6001 group at four weeks after implant placement indicated that MMP inhibitor GM6001 is effective in altering the MMP-influenced control of matrix remodeling at the implant bone interface.

The present findings have implications for the field of bone implants. The loss coefficient gives a direct indication of implant stability via an analysis of the response to percussion loading. The known role of MMPs in bone healing as well as histological examination of CD40 protein at the bone/implant interface indicate that the extent of osseointegration can be clinically evaluated from loss coefficient data.

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Figure 1.
The approach position of the percussion probe onto the testing abutment is shown. The supporting arm is not in contact with the animal's head.

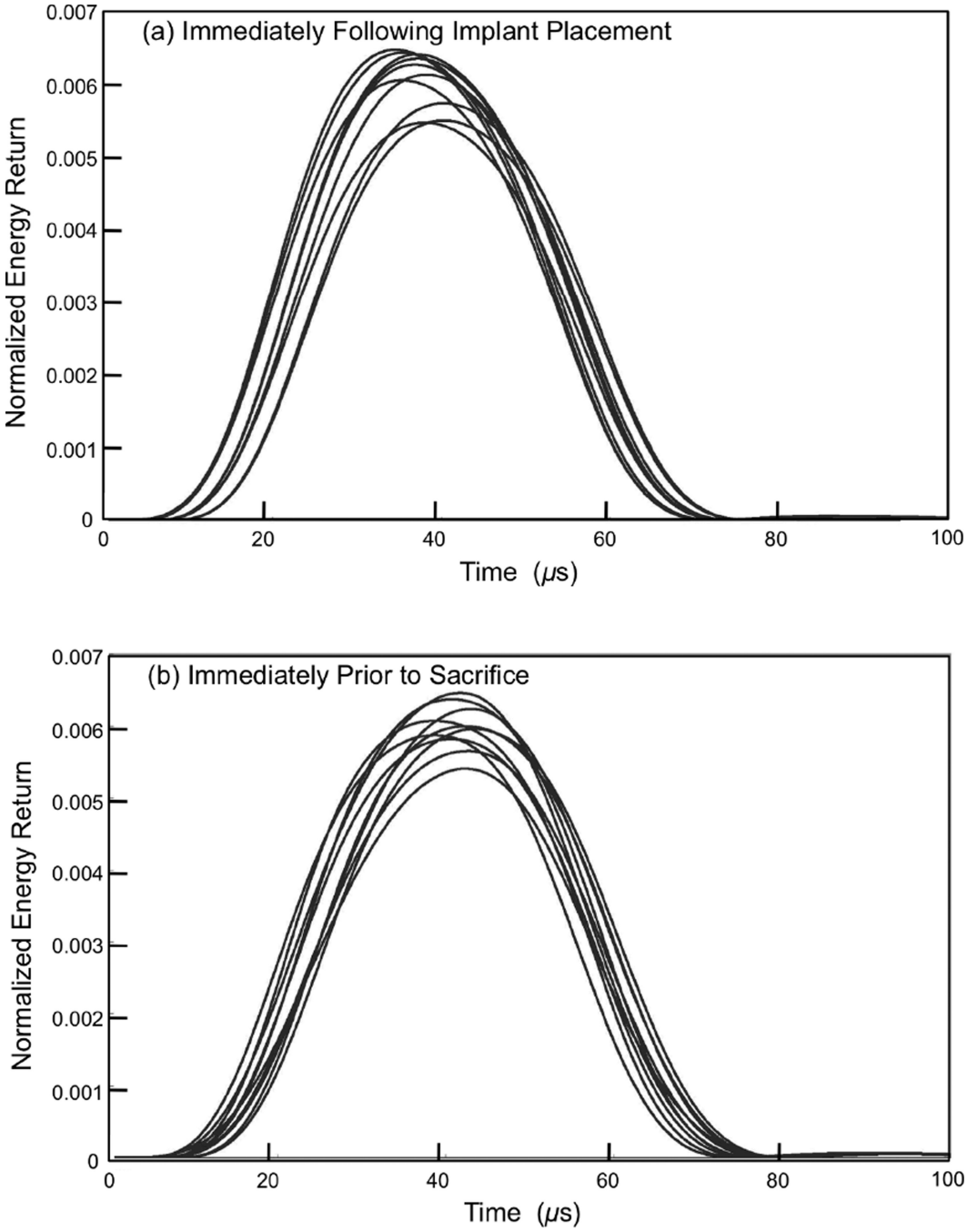

Figure 2.

Energy return data corresponding to ten individual percussions for a 2-week vehicle animal at (a) surgical placement and (b) sacrifice that are representative of the entire study. The energy return profiles for all animal subjects were uniformly shaped at each time point, as shown here, with no additional peaks indicating there were no significant defects at the bone/implant interface.

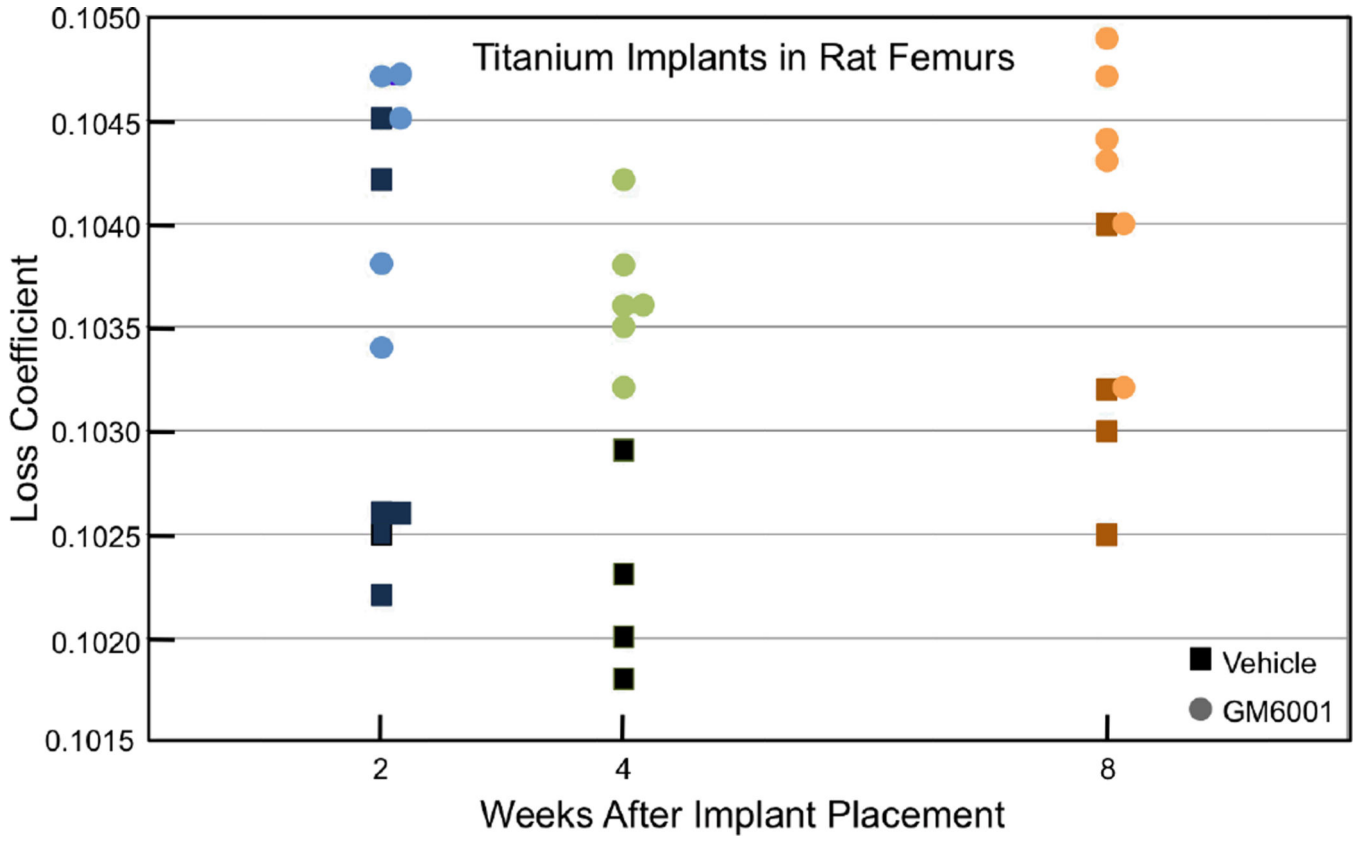

Figure 3.

Loss coefficient values at each therapy time point with replicate values plotted slightly to the right of the corresponding time point. The square symbols mark vehicle group data and the slightly lighter circular markers indicate GM6001 data.

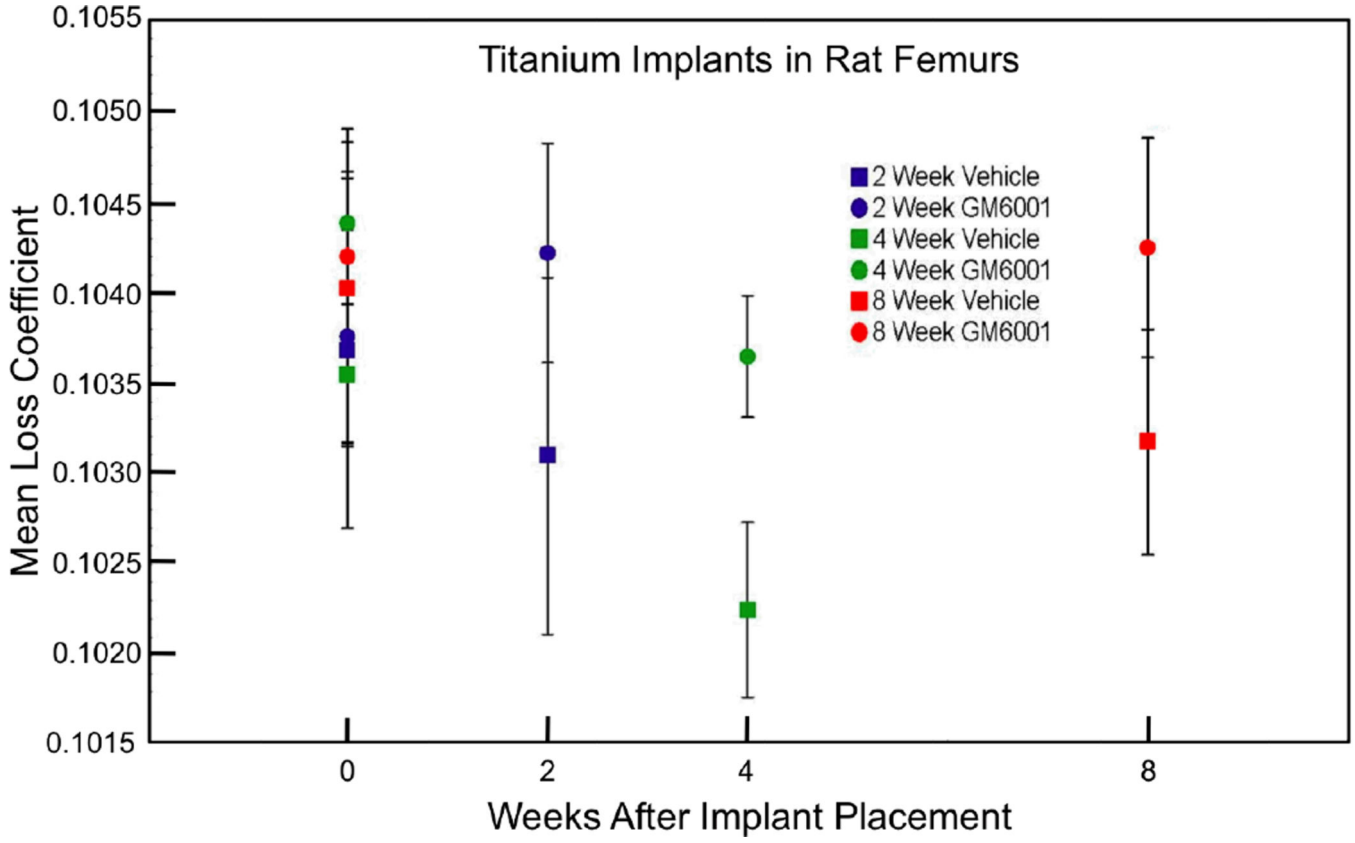

Figure 4.

Mean LC values for all therapeutic groups from placement to sacrifice. Error bars shown correspond to the standard deviation for each mean. The data for the 4-week duration group is particularly striking and proved statistically significant. The 2-week sacrifice data demonstrated significant difference from placement values, but not between therapy groups.

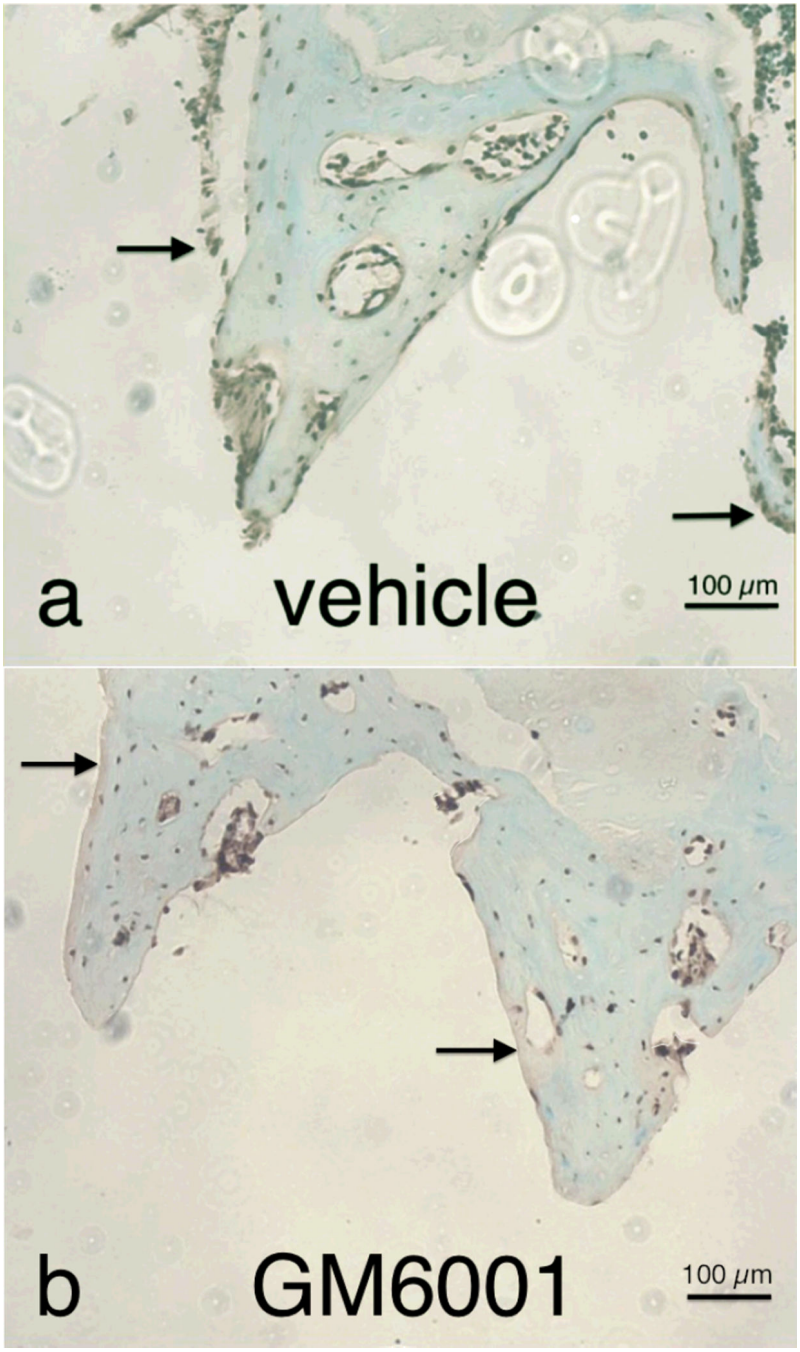

Figure 5.
Images of the bone adjacent to an implant from 4 week duration groups showing the difference in CD40 localization between the (a) vehicle group, and (b) GM6001 treated group.

Table 1

Analysis of Loss Coefficient Means

Time to Sacrifice	Comparison being Tested	No. of Cases	*t*-Test Type	*t*-Score	Two-Sided p-value
2 Weeks	GM6001 LC: Placement vs Sacrifice	5	Paired	0.93	0.405
	Vehicle LC: Placement vs Sacrifice	6	Paired	−1.14	0.306
	LC at Sacrifice: Vehicle vs GM6001	11	Independent	2.22	0.053
4 Weeks	GM6001 LC: Placement vs Sacrifice	6	Paired	−3.84	0.012
	Vehicle LC: Placement vs Sacrifice	4	Paired	−4.75	0.018
	LC at Sacrifice: Vehicle vs GM6001	10	Independent	5.50	0.001
8 Weeks	GM6001 LC: Placement vs Sacrifice	6	Paired	0.16	0.881
	Vehicle LC: Placement vs Sacrifice	4	Paired	−1.29	0.288
	LC at Sacrifice: Vehicle vs GM6001	10	Independent	2.73	0.026