OSSEOINTEGRATION OF TEMPORARY ANCHORAGE DEVICES USING
RECOMBINANT HUMAN BONE MORPHOGENETIC PROTEIN-2

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Erin E. Cruz

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COMMITTEE MEMBERSHIP

TITLE: Osseointegration of Temporary Anchorage Devices
Using Recombinant Human Bone Morphogenetic
Protein-2

AUTHOR: Erin E. Cruz

DATE SUBMITTED: May 2010

COMMITTEE CHAIR: Lanny V. Griffin, Dept Chair-Biomed & Gen Eng

COMMITTEE MEMBER: Daniel W. Walsh, Assoc Dean Grad Prgm/Rsrch

COMMITTEE MEMBER: Robert B. Szlavik, Instr Fac AY

ABSTRACT

Osseointegration of Temporary Anchorage Devices Using Recombinant

Human Bone Morphogenetic Protein-2

Erin E. Cruz

Over the past 5 years, the use of titanium implants as temporary anchorage devices (TADs) has become an important tool in clinical orthodontic practices. The use of TADs have provided orthodontists a way of moving teeth against fixed objects rather than against the surrounding teeth, which tend to counteract desired motion. At present, viable attachment of TADs involves direct insertion through gingival tissue and piercing of the bone. Surface modifications such as sandblasted and acid-etched treatment or bone morphogenetic protein surface treatment, however, can be applied to the TADs to promote enhanced osseointegration, thereby allowing the TADs to serve as stable anchors while avoiding bone puncture. In this study, a comparison was made between sandblasted/acid-etched TADs and sandblasted/acid-etched/recombinant human bone morphogenetic protein-2 (rhBMP-2) treated TADs to determine whether rhBMP-2 promotes enhanced osseointegration. A total of 10 rats (4 controls and 6 treated with rhBMP-2) were used in the study, with 1 TAD placed on the skull of each rat. At the end of 6 weeks, the animals were euthanized by carbon dioxide asphyxiation, and bone blocks, each containing a TAD, were prepared for histological examination and biomechanical characterization. The results of this study showed that TADs treated with rhBMP-2 had greater bone formation at the bone-implant interface and an increase in total implant stability.

Keywords: recombinant human bone morphogenetic protein-2 (rhBMP-2), osseointegration, temporary anchorage device, sandblasted and acid etched, titanium

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1. LITERATURE REVIEW

For more than 40 years, titanium has been widely used as a biocompatible implant material for dental applications. In general and prosthetic dentistry, titanium implants today have become a regular and customary alternative to one or more missing teeth. Orthodontics, however, has been slow in using this important tool as a temporary anchor. It has only been in the last 5 years that an explosion of temporary anchorage devices (TADs) has appeared in clinical orthodontic practices.

During orthodontic treatment, TADs control tooth movement by preventing unwanted or allowing desired dental movement [1]. In orthodontics, the difficulty that usually arises when moving teeth is that the surrounding teeth tend to counteract the desired motion. This is consistent with Newton's third law, which states that for every action, there is an equal and opposite reaction. However, instead of using molars as anchors, which may facilitate movement of the teeth (action / reaction forces) as defined by Newton's third law, the use of TADs allows teeth to be moved against fixed objects rather than against the surrounding teeth. Therefore, TADs provide orthodontists and patients with appealing treatment alternatives to restorative and surgical procedures.

Although TADs show great potential in orthodontic practice, they also introduce possible serious risks to the anatomical structures of the maxillary and mandibular bones. If there is inadequate amount of bone available at the implant site or insufficient space to insert TADs that are 5 to 12 mm in length and 1.3 to 2 mm in diameter, then the roots of the adjacent teeth, soft tissue, sinus and nasal cavities, and neurovascular bundles could

be damaged [2, 3]. At present, viable attachment of TADs involves direct insertion through the gingival tissue and piercing of the bone. Unfortunately, bone puncture may induce bone infections, such as localized or systemic osteomyelitis. In addition, when considering the roots of the teeth, a bone piercing TAD would be limited in terms of insertion site. With a bone piercing TAD, the TAD would have to be placed between the roots in order to avoid injury to the roots or other anatomical structures nearby. With this in mind, would it be possible to get the undersurface of a TAD to adhere to bone and thus avoid the undesirable side effects associated with bone piercing?

In 1995, Block and Hoffman developed a disc-like structure called an “onplant,” which required less bone depth compared to the usual endosseous (contained within bone) implants. The onplant is a hydroxyapatite-coated titanium alloy disk that is 10 mm in diameter and 2 mm high (refer to Fig. 1) and placed on the palatal bone to provide anchorage for orthodontic tooth movement [3]. Through experimentation, Block and Hoffman found that the onplant, after subjected to orthodontic forces, provided total anchorage for tooth movement without reciprocal movement of the onplant [3, 4]. In addition, the onplant prevented anterior dental retraction by providing maximum anchorage to molars [3, 4]. However, a disadvantage of the onplant is the complications that occur with the removal process. During the removal of the onplant, a large portion of soft tissue must be re-exposed, which could be uncomfortable for the patient. Although the onplant takes bone height into account and avoids the risks involved with bone perforation, the removal technique introduces invasive procedures. Would it be possible to avoid piercing the bone and develop a removal technique that is less-invasive?

Surface TADs could provide a way to minimize the discomfort associated with the removal process while avoiding puncture of the bone. However, surface TADs introduce limitations as well. Compared to longer and wider implants, surface TADs make it more difficult to achieve mechanical retention. Knowing this, how could the bone-to-TAD interface be strengthened in order to attain the mechanical retention desired?

Figure 1-1 – Picture of an Onplant

Taken from *The Role of Implants in Orthodontic* by S.F.H. Ismail and A.S. Johal

Per Ingvar Branemark first coined the term osseointegration when a titanium cylinder used in one of his experiments had integrated tightly into rabbit bone. More specifically, osseointegration is defined as the direct structural and functional connection between living bone and the surface of an implant. Bone-compatible materials such as titanium, titanium alloys, and hydroxyapatite, have been shown to achieve osseointegration [4]. Presently, osseointegrated implants are used to treat both completely edentulous (being without teeth) and partially edentulous ridges. In addition, osseointegration makes it possible to increase orthodontic anchorage because an implant directly integrated into bone should not move in response to orthodontic loads [4]. Therefore, through osseointegration, a TAD made of titanium could serve as a stable anchor.

For this study, titanium is chosen as the dental implant material because it has excellent biocompatibility, is strong and lightweight, and is highly resistant to corrosion, stress, and fracture [5, 6, 7, 8]. Titanium has been used for both endosseous and subperiosteal implants for many years. In addition, titanium easily passivates to form a protective oxide layer, which allows the apposition of proteins and tissue to the metal surface [7]. Thus, titanium itself promotes osseointegration because direct contact occurs between the bone and the implant surface [7, 9]. Although other materials, such as gold and platinum, have shown to be corrosion resistant, their low tensile strength makes titanium a better choice of implant material. Furthermore, stainless steel has also been used in orthodontic practice because of its resistance to corrosion and its high strength. Its high corrosion resistance is due to its low carbon and sulfur content and its high chromium and nickel content. Unfortunately, nickel is not a biocompatible material and has shown to cause allergic reactions [5]. On the other hand, titanium is well tolerated by the human body. Therefore, titanium's physical and mechanical properties, along with its ability to integrate into bone, make it an advantageous implant material.

According to previous studies, the surface of an implant has been a determining factor for successful osseointegration (direct bond between living bone and the surface of an implant), and in particular, microrough titanium surfaces have facilitated enhanced bone formation and implant stability [9, 10, 11, 12, 13]. The combination of a sandblasted and acid-etched surface has improved bone apposition [9, 10, 12] and resulted in higher torque values during biomechanical testing [10, 11, 12]. What if,

however, it was possible to increase osseointegration even more? This could lead to enhanced implant coverage by bone as well as an increase in adhesion strength at the bone-to-implant interface. Therefore, if appropriate surface modifications can be applied to the TADs to promote enhanced osseointegration, the TADs could serve as stable anchors while avoiding puncture of the bone. With enhanced osseointegration, the TADs could develop higher levels of mechanical resistance and stability.

One type of surface modification involves the use of bone morphogenetic protein (BMP). BMPs belong to a family of growth factors and are known for their pivotal role in cartilage and bone formation [14, 15, 16, 17, 18]. For decades, BMPs have been studied because of their ability to heal bone while reducing or eliminating the need to harvest bone from other parts of the body. So far, a total of 20 BMPs have been discovered, eight (BMP-2 through BMP-9) of which are members of the transforming growth factor- β (TGF- β) superfamily of proteins. Of these eight, BMP-2 and BMP-7 have gained the most attention in regards to therapeutic procedures to treat a variety of skeletal conditions and defects. BMP-2 and BMP-7 have demonstrated the greatest osteogenic capacity by influencing the differentiation of mesenchymal cells to chondroblasts and osteoblasts [16]. Experimental studies have shown the importance of BMP-2 and BMP-7 in bone development and the development of a variety of tissues outside of bone [14, 17].

By understanding the important role that BMP plays in cell growth and differentiation, scientists have been able to create a genetically engineered version of this naturally occurring protein called recombinant human bone morphogenetic protein

(rhBMP). More importantly, a large number of preclinical studies have verified the ability of rhBMPs to initiate bone growth [16, 17, 18, 19, 20]. In particular, rhBMP-2 has been studied more than any other BMP and has received Food and Drug Administration (FDA) approval for human clinical uses, such as oral, spinal, and tibial surgeries. The local application of rhBMP-2, in addition to a variety of carriers such as a collagen sponge, has demonstrated the ability to regenerate skull and mandibular defects [15, 17], enhance periodontal new bone formation [15, 16], improve spinal fusion [15, 16, 18, 19, 21], treat a variety of bone-related conditions such as delayed union and non-union [18], and promote fracture repair [15, 18, 19].

For this present study, rhBMP-2 is chosen because of its effectiveness in stimulating bone growth in a variety of applications. Specifically, INFUSE® Bone Graft (Medtronic Sofamor Danek, Memphis, TN), which consists of rhBMP-2 in combination with an absorbable collagen sponge (ACS), is used. Medtronic INFUSE® Bone Graft has helped in the spine by promoting the fusion of vertebral bodies to alleviate back pain. It has also helped to heal broken bones for tibial fractures, and been used for oral and maxillofacial bone grafting procedures, such as sinus augmentation and localized alveolar ridge augmentation. Even though studies have validated the effectiveness of the rhBMP-2/ACS in stimulating new bone growth, it is still important to participate in further studies which will expand knowledge of its use and application.

For rhBMP-2 to enhance osseointegration, the implant must be stabilized. Without implant stability, there is a risk of microfractures at the implant-bone interface,

which could decrease the likelihood of osseointegration. Therefore, it is important for TAD stability to exist before and until osseointegration can be obtained. When a simple screw TAD is threaded into bone, mechanical resistance can serve as the necessary stabilizer until osseointegration occurs. Mechanical resistance and stability is achieved because when a force is applied to a body (i.e. tooth) to the left and produces a counterclockwise moment about a center of resistance (i.e. screw TAD), an opposite system counteracts with a force to the right and a moment in the clockwise direction [4]. This means that the sum of all forces equals zero, and the sum of all moments about a point is also zero. Thus, the screw TAD achieves static equilibrium.

However, when the goal is to avoid bone puncture by surface osseointegration, what means of stabilization can be employed to ensure that the TAD is stabilized until osseointegration can be achieved? Since the Vietnam War, cyanoacrylate glues have been used in medical procedures to seal soft tissue wounds. There are a variety of cyanoacrylates, and each is appropriate for different applications. For instance, methyl cyanoacrylates have the most rigid polymer matrix and are excellent for metal-bonding applications. On the other hand, ethyl cyanoacrylates work well on plastics and elastomeric substrates [22]. Butyl cyanoacrylate, in particular, has been tolerated by mammalian tissues and used as a surface dressing at oral wounds of both animals and human beings [23]. In addition, butyl cyanoacrylate has also shown to reduce edema and cause minimal tissue response. Using cyanoacrylate to adhere TADs to the bone is very important to the success of this study because achieving TAD stability is essential for osseointegration to be obtained.

2. RESEARCH & SPECIFIC AIMS

2.1 Objective

The goal of this study is to examine the viability of a combination of existent technologies, which include temporary anchorage devices (TADs), sandblasted and acid etched surface treatment, bone morphogenetic protein surface treatment, and cyanoacrylates. In particular, the goal is to evaluate the effect of recombinant human bone morphogenetic protein-2 (rhBMP-2) on the ability of TADs to osseointegrate. A comparison will be made between sandblasted/acid-etched TADs and sandblasted/acid-etched/rhBMP-2 treated TADs to determine whether rhBMP-2 promotes enhanced osseointegration. Specifically, this research will determine the influence of Medtronic INFUSE® Bone Graft on implant anchorage and increased bone formation. Finding a way to enhance bone formation and strengthen bone-to-TAD contact (increased implant stability) is important because there is a significant clinical benefit to patients if a new type of TAD that does not pierce the bone is available. Not only can teeth movement be controlled without causing movement to the surrounding teeth, but enhanced bone formation at the implant site can prevent bone puncture and serious side effects.

2.2 Specific Aim

To investigate the influence of recombinant human bone morphogenetic protein-2 (rhBMP-2) on the osseointegration of temporary anchorage devices

(TADs). This will involve qualitatively examining surface topography and the amount of the TAD surface covered by bone through photographs and visual inspection. The bone formation on a sandblasted/acid-etched/rhBMP-2 treated TAD will be compared with bone formation on a sandblasted/acid-etched control specimen. In addition, biomechanical testing will be done using a micromechanical testing system to determine if the rhBMP-2 treatment on the TAD produces any significant changes in the bond strength between the TAD and the bone.

2.3 Hypothesis

Sandblasted and acid-etched temporary anchorage devices (TADs) treated with recombinant human bone morphogenetic protein-2 (rhBMP-2) will promote better osseointegration than TADs that are only sandblasted and acid-etched. Specifically, TADs treated with rhBMP-2 on top will show an increase in bone formation (implant surface covered by bone) and an increase in total implant stability (adhesion strength at the bone-to-TAD interface).

3. MATERIALS & METHODS

3.1 Subjects

3.1.1 Guidelines

Ten adult Sprague-Dawley rats (4 controls and 6 treated with the absorbable collagen sponge/rhBMP-2 mixture) with an average weight of 450 ± 70 grams were used in this study. The rats were handled in accordance with National Institutes of Health (NIH) guidelines for the care and use of laboratory animals (NIH publication 85-23, Rev. 1985) and the surgeries were performed with the approval of the Animal Care and Use Committee (IACUC) of California Polytechnic State University, San Luis Obispo. The details of each rat surgery (i.e. time of injection, start time of surgery, end time of surgery, etc.) were recorded in a lab notebook. A copy of this raw data is found in Appendix A.

3.1.2 Rationale for Species and Numbers

The Sprague-Dawley rat was selected as the appropriate species choice for this study due to its genetic similarity to humans and its ability to serve as a general model for the study of human health and disease. Sprague-Dawley rats are widely used for experimental purposes, and in particular, their calmness and ease of handling make them the choice of species for this study. Studies similar to this

current study have shown sound scientific results with the use of Sprague-Dawley rats.

Using a 1-Sample t-test, 10 rats (4 controls and 6 treated with the absorbable collagen sponge/rhBMP-2 mixture) were chosen for this study so that a sufficient amount of data is produced while limiting the number of rats to be used.

3.2 Implant Preparation

The temporary anchorage devices (TADs) used in this study are manufactured from commercially pure titanium and are divided into 2 types of treatments: Type 1 is sandblasted (with grit) and acid-etched (combination of anorganic acids, HCl/H₂SO₄ acid bath), and Type 2 is treated with Medtronic INFUSE® Bone Graft (recombinant human bone morphogenetic protein-2 placed on an absorbable collagen sponge) in addition to being sandblasted and acid-etched. For a type 1 treated TAD, a 1 cm x 1 cm absorbable collagen sponge with 0.09cc of saline will be placed on top of the sandblasted and acid-etched implant to serve as the control. Refer to Figures 3-1 and 3-2 below.

Figure 3-1 – Wetting 1 cm x 1 cm Collagen Sponge with 0.09cc of Saline

Figure 3-2 – Wetted Collagen Sponge Placed on Top of Control TAD

For a type 2 treated TAD, a 1 cm x 1 cm absorbable collagen sponge with 0.09 cc of reconstituted rhBMP-2 will be placed on top of the TAD to serve as the rhBMP-2 treated TAD. The collagen sponge with saline is used so that the only variable between the type 1 TAD and the type 2 TAD is the reconstituted rhBMP-2. The collagen sponge must be soaked in the saline or rhBMP-2 for at least 15 minutes before it is used.

3.3 Surgical Procedures

3.3.1 General

During preliminary experimentation, one rat was used to evaluate the following: 1) Effectiveness of the anesthesia (Ketamine, Xylazine, Buprenorphine), 2) Instrumentation needed to perform the surgical procedure, 3) Pre-operative, operative, and post-operative guidelines, 4) Type and location of incision, 5) Implant contour, adhesion method, closure of incision site, and 6) Implant size and protruding

length. Details of this preliminary experiment can be found in Appendix B. The actual surgical procedures performed for this study were completed based on the findings from the preliminary experiments. In addition, a Surgical Checklist which is found in Appendix C was created to ensure that all materials and instruments were in the surgical suite at the time of surgery, and that no procedural steps were missed.

3.3.2 Pre-Operative

The instruments used during the procedure were sterilized by heat sterilization prior to prep and surgery. The following instruments were sterilized: a tray for rhBMP-2 procedures, forceps, fine forceps, ultrafine forceps, scissors, scalpel, butyl cyanoacrylate applicator, glass slab for butyl, surgical drapes, syringes, gloves, Petri dish with saline, cotton gauze, and cotton swabs. Once the instruments were sterilized, they were brought to the surgical suite along with other materials/equipment such as butyl cyanoacrylate, tupperware for weighing the rat, isolation mask and cap, sterile gowns, bench covers, weight boat, and the rat in its cage.

Once all the instruments and equipment were available in the surgical suite, the assistant prepared the absorbable collagen sponge (ACS) for use in surgery. In preparation for surgery that used a control TAD,

0.09cc of saline was placed on a 1cm x 1 cm ACS using a syringe and sterile tray. Likewise, in preparation for surgery that used an rhBMP-2 treated TAD, 0.09cc of reconstituted rhBMP-2 was placed on a 1cm x 1 cm ACS using a syringe and sterile tray. The collagen sponge was soaked in the saline (or rhBMP-2) for at least 15 minutes.

Preliminary experiments were done to determine the appropriate size of ACS and the amount of rhBMP-2 required for each implant, as well as the best tool for cutting the sponge and the best method for shaping the sponge during the procedure. Details of these experiments can be found in Appendix D.

While the ACS soaked in either saline or rhBMP-2, the rat was weighed to determine the appropriate dosages of Ketamine, Xylazine, and Buprenorphine to be administered to the rat. The rat was placed on a bench cover and a mixture of Ketamine and Xylazine (44 mg/kg body weight of Ketamine, 10 mg/kg body weight of Xylazine) was used for inducing general anesthesia. This provided approximately 30 minutes of effective anesthesia. A ½ dose was readily available for surgeries that took longer than expected. The source used to determine these dosages was Lumb and Jones Veterinary Anesthesia and Analgesia, 4th Ed. by W. J. Tranquilla. In addition, a dose of Buprenorphine was administered subcutaneously (0.01 – 0.05 mg/kg body weight) between the shoulder blades for pain mitigation [24].

The rat was then placed in a chamber until the anesthesia took effect and the animal was down. The table in Appendix E shows the number of mL of each drug that should be administered based on the rat's weight. The dosages were recorded in a lab notebook which is under lock and key at the California Polytechnic State University, San Luis Obispo surgical suite. The Ketamine/Xylazine and Buprenorphine mixture works well because emergence from anesthesia is smoother and has a high factor of safety. Under anesthesia, the rat did not respond to various audible and tactile stimuli, and maintained a normal breathing and heart rate. Once the anesthesia had taken effect and the rat was in Stage 3, it was taken out of the chamber to undergo prep procedures.

The assistant prepped the rat by shaving the surgical site (rat's head), applying depilatory cream to remove excess hair, and disinfecting the area with Betadine solution using sterile cotton swabs applied in a circular motion. This process was performed 3 times (with 3 different cotton swabs) and the antiseptic was in contact with the skin for a minimum of 3 minutes before incision. The rat was put back in the chamber while the surgical area was prepped.

The surgical area was prepped by disinfecting the table to maintain a clean, uncluttered, and sanitized area. A new bench cover was placed

on the table and the rat was placed on top of it. To prep for post-operative procedures, the assistant also placed a heating pad in the microwave so that it was ready to heat up once the surgical procedures were completed. Once the “surgeon” had scrubbed and put on the sterile gown, he/she put on the sterile gloves and sat down to begin surgery. The assistant carefully opened the pre-sterile package containing the sterile surgical drape and the surgeon removed the drape from the packaging and placed it over the rat to ensure that aseptic standards were met. With the help of the assistant, the other sterile packages containing the sterile instruments, butyl cyanoacrylate/applicator, glass slab, gauze, and cotton swabs were also opened while the surgeon removed the contents of those sterile packages and placed them onto the sterile field. The rat was ready to undergo the surgical procedure.

3.3.3 Operative Procedures

Using the “tips-only” technique, the surgeon made an incision approximately 1 inch in length along the midline of the head from the eyes to the ears using firm pressure to guarantee a clean cut in a single stroke. Once the incision was made, the periosteum was carefully folded back to expose the bony complex on the surface of the cranium. Sterile forceps were used to pick up one temporary anchorage device, contour it to fit the shape of the animal’s skull, and place it on the

surface of the cranial bone. Preliminary experiments showed that only one 7mm diameter TAD can be successfully placed on the skull and covered with the periosteum. The periosteum is too thin to effectively cover more than one implant. In addition, preliminary experiments confirmed that a protruding length of 2mm on one side of the TAD is adequate for biomechanical testing but small enough to prevent the rat from knocking it off.

The implant was then attached to the bone using the tissue glue, butyl cyanoacrylate, which took approximately 2 minutes to set. The TAD was placed on the cranial bone such that its distance from the cranial surface was as close as possible, allowing for enhanced bone apposition and likelihood of osseointegration. Butyl cyanoacrylate was evaluated during preliminary experiments and was shown to be an effective adhesive. It was also determined through preliminary experimentation that although the curing time with UV light is less than the curing time without UV light, the difference is not significant enough to require the use of UV light for this study. Refer to Appendix F for details and results of these preliminary experiments.

For both the control and the rhBMP-2 treated TADs, sterile forceps were used to place the wetted collagen sponge on top of the implant. The collagen sponge was wet with saline or reconstituted rhBMP-2

depending if it was a control implant or not. While surgery was in progress, the animal's vital signs were continually monitored. Once the TAD was stable, the periosteum was folded back over the implant. Sterile forceps were used to hold the sides of the skin at the incision site together while the incision was closed using butyl cyanoacrylate. This completed the surgery. The circulating heating pad was then heated up in the microwave for about 3-4 minutes to heat up to 37 degrees.

3.3.4 Post-Operative

After surgery and the removal of the surgical drape, the rat was given a subcutaneous injection of Buprenorphine (0.01 – 0.05 mg/kg). The rat was then moved to a warm area (recovery bin) where it was monitored during recovery. A heating pad was used to return the animal's body temperature back to normal. Upon full recovery from the anesthesia, the rat was returned to its routine housing; each rat was caged separately. Buprenorphine was administered 3 times a day for 2 days as necessary for pain management. The rats were visually inspected daily post-op for infection, and a surgical record (procedure, date, anesthesia dose, route of administration) was maintained.

3.4 Histological Examination

The rats were euthanized by carbon dioxide asphyxiation and a qualitative analysis of surface topography and the amount of the TAD surface covered by bone was conducted through photographs and visual inspection. A comparison of surface topography and TAD coverage was made between the sandblasted/acid-etched/rhBMP-2 treated TAD and the control specimen (sandblasted and acid-etched TAD). The results of the histological examination are found in Section 4.1 below.

3.5 Biomechanical Testing

3.5.1 General

After qualitative analysis, biomechanical testing was performed using the In Spec, Instron Corporation, Camden, MA micromechanical testing system. Load was applied to the upper part of each temporary anchorage device in the shear direction with a force at constant speed until the surrounding bone was destroyed.

3.5.2 Experimental Model

As a proof of experimental concept, the TAD was imbedded onto a polyurethane 20 lb foam block and attached to the In Spec, Instron Corporation, Camden, MA micromechanical testing

system with a 50 N load cell. Five replicate tests were conducted with an average shear load of $8.9 \text{ N} \pm 1.5 \text{ N}$.

Figure 3-3 – Experimental Model for Biomechanical Testing

3.5.3 Test Setup for Biomechanical Testing

Figure 3-4 – Biomechanical Testing Setup

Figure 3-5 – Applying Load to Implant

4. RESULTS

4.1 Histological Examination Results Summary

Visual inspection of the TADs prior to biomechanical testing showed bone formation on both the control TADs and the TADs treated with rhBMP-2. However, TADs treated with rhBMP-2 had a greater amount of bone coverage (enhanced osseointegration) than the control TADs. For all but one of the rhBMP-2 treated TADs (exception is Rat # 10), only the protruding portion of the TAD was visible after 6 weeks of implantation in the rat's skull as the top surface of the TAD was almost entirely covered in new bone. For Rat #10, the TAD site was infected and thus had minimal to no bone coverage. Table 4-1 below shows the percentage of bone coverage observed on each TAD for each rat. The raw data (TAD photographs) can be found in Appendix G.

Table 4-1 – Percent Bone Coverage on TAD

Rat #	Control or rhBMP-2 treated	Percent Bone Coverage
Rat 5	Control, sponge on top	10%
Rat 6	Control, sponge on top	0% – TAD migrated
Rat 7	Control, sponge on top	N/A - rat died after surgery
Rat 9	Sponge on top w/ rhBMP-2	99%
Rat 10	Sponge on top w/ rhBMP-2	0% - TAD site is infected
Rat 11	Sponge on top w/ rhBMP-2	90%
Rat 19	Sponge on top w/ rhBMP-2	N/A– rat died a few days after surgery
Rat 21	Control, sponge on top	10%
Rat 22	Sponge on top w/ rhBMP-2	99%
Rat 23	Sponge on top w/ rhBMP-2	99%
Rat 24	Control, sponge on top	5%
Rat 26	Sponge on top w/ rhBMP-2	N/A - Rat died one day after surgery
Rat 27	Sponge on top w/ rhBMP-2	99%

Based on the data in Table 4-1 above, the average percentage of bone coverage on a control TAD was $6.25 \pm 4.79\%$ while the average percentage of bone coverage on an rhBMP-2 treated TAD was $97.2 \pm 4.02\%$ (Figure 4-1).

Figure 4-1 – Avg. Percent Bone Coverage & Stdev

4.2 Biomechanical Testing

4.2.1 Results Summary

The biomechanical testing results showed that TADs treated with the rhBMP-2 on top have a significantly greater failure load than the control TADs. The failure load for the rhBMP-2 treated TADs ranged from 32.02 N – 98.39 N, whereas the failure load for the control TADs ranged from 0 N – 5 N. The value of 0.96 N for rat #10 was excluded as the TAD site was found to be infected and thus inconclusive. See details in Table 4-2 below.

Table 4-2 – Failure Load Data for Each Rat

Rat #	Control or rhBMP-2 treated?	Failure Load
Rat 5	Control, sponge on top	Data lost but failure load observed on hand-held device was ≈ 5 N
Rat 6	Control, sponge on top	0 N - No testing performed as TAD migrated during the 6 weeks
Rat 7	Control, sponge on top	N/A – rat died after surgery
Rat 9	Sponge on top w/ rhBMP-2	88.16 N
Rat 10	Sponge on top w/ rhBMP-2	0.96 N – TAD site is infected
Rat 11	Sponge on top w/ rhBMP-2	32.02 N
Rat 19	Sponge on top w/ rhBMP-2	N/A – rat died after surgery
Rat 21	Control, sponge on top	4.99 N
Rat 22	Sponge on top w/ rhBMP-2	98.39 N
Rat 23	Sponge on top w/ rhBMP-2	92.92 N
Rat 24	Control, sponge on top	2.84 N
Rat 26	Sponge on top w/ rhBMP-2	N/A – rat died one day after surgery
Rat 27	Sponge on top w/ rhBMP-2	68.14 N

4.2.2 Mean Failure Load & Standard Deviation

Based on the data in Table 4-2 above, the mean failure load for the control TADs is $3.21 \text{ N} \pm 2.37 \text{ N}$ while the mean failure load for the rhBMP-2 treated TADs is $75.93 \text{ N} \pm 27.07 \text{ N}$ (Figure 4-2 below).

Figure 4-2 – Mean Failure Load & Standard Deviation

The failure load graphs for each rat/TAD are found in Appendix H.

5. DISCUSSION OF RESULTS

5.1 Bone Coverage on TAD

Bone formation was observed on both the control TADs and the TADs treated with rhBMP-2. Control TADs had a percentage of bone coverage of $6.25 \pm 4.79\%$. For 3 of the 4 control TADs, bone coverage was present as a result of its sandblasted and acid-etched surface. As described in the Literature Review section of this paper, the combination of a sandblasted and acid-etched surface has been known to improve bone apposition. Based on the results, this type of microrough titanium surface does in fact facilitate the formation of new bone.

For 1 of the control TADs, however, no bone coverage was observed as the implant had migrated sometime during the 6 week implantation period. The reason for implant migration may be due to one or a combination of the following: 1) the TAD was knocked loose during the 6 week implantation period (i.e. the rat knocked it out of place), 2) not enough butyl cyanoacrylate was used to glue the TAD to the bone during the rat surgery resulting in an unstable TAD, and 3) not enough butyl cyanoacrylate was used to adequately seal the surgical incision making it easier for the rat to knock the TAD out of place. Without TAD stability, the risk of microfractures at the TAD-bone interface increases which in turn decreases the likelihood of osseointegration. See Figure 5-1 below for the percent bone coverage summary for a control TAD.

Figure 5-1 – Percent Bone Coverage Summary for Control TAD

Even further, the TADs which were sandblasted, acid-etched, and treated with rhBMP-2, showed even more bone coverage (enhanced osseointegration) than the control TADs. For all but one of the rhBMP-2 treated TADs (exception is Rat # 10), only the protruding portion of the TAD was visible after 6 weeks of implantation in the rat's skull as the top surface of the TAD was almost entirely covered in new bone. Specifically, the rhBMP-2 treated TADs had a percentage of bone coverage of $97.2 \pm 4.02\%$. Compared to a control TAD, the percent bone coverage on an rhBMP-2 treated TAD was significantly greater. This shows that rhBMP-2 promotes enhanced osseointegration. The significance of rhBMP-2 on enhancing bone formation was quantified during biomechanical testing which is discussed in Section 5.2 below. See

Figure 5-2 below for the percent bone coverage summary for an rhBMP-2 treated TAD.

Figure 5-2 – Percent Bone Coverage for an rhBMP-2 treated TAD

As referenced above, although the TAD in Rat #10 was treated with rhBMP-2, visual inspection of the TAD prior to biomechanical testing showed minimal to no bone formation compared to the rest of the rhBMP-2 treated TADs. This TAD site was found to be infected, which likely interfered with bone formation in this area.

5.2 TAD Stability

The biomechanical testing results showed that TADs treated with the rhBMP-2 on top have a significantly greater failure load than the control TADs. The failure load for the rhBMP-2 treated TADs ranged from 32.02 N – 98.39 N, whereas the failure load for the control TADs ranged from 0

N – 5 N. The value of 0.96 N for rat #10 was excluded from the calculation and analysis for the rhBMP-2 treated TADs as the TAD site was found to be infected and thus inconclusive. The large range for the rhBMP-2 treated TADs is largely due to TAD stability (i.e. how well the butyl cyanoacrylate glues the TAD to the bone prior to osseointegration or how well the TAD contours to the rat’s skull). The more stable the TAD, the better chance for enhanced osseointegration. On average, the amount of force required to pull an rhBMP-2 treated TAD from the bone was 75.93 N $\pm$ 27.02 N. See Figure 5-1 below for the failure load summary for an rhBMP-2 treated TAD. This average does not include the data for Rat #10.

Figure 5-3 – Failure Load Summary for rhBMP-2 Treated TAD

In contrast, the average amount of force required to pull a control TAD from the bone was $3.21 \text{ N} \pm 2.37 \text{ N}$. See Figure 5-2 below for the failure load summary for a control TAD. This average includes the data for Rat #6, which is 0 N since the TAD migrated sometime during the 6 week timeframe. If 0 N was removed as a data point based on the assumption that osseointegration would have occurred had the TAD been stable, then the average failure load would be $4.23 \text{ N} \pm 1.24 \text{ N}$.

Figure 5-4 – Failure Load Summary for Control TAD

6. CONCLUSIONS

Based on the visual inspection and biomechanical testing results, sandblasted and acid-etched temporary anchorage devices (TADs) treated with recombinant human bone morphogenetic protein-2 (rhBMP-2) promote better osseointegration than TADs that are only sandblasted and acid-etched. Specifically, TADs treated with rhBMP-2 showed an increase in bone formation (avg. of 97.2% TAD surface covered by bone vs. avg. of 6.25% TAD surface covered by bone) and an increase in total TAD stability, or adhesion strength at the bone-to-TAD interface (avg. of 75.93 N vs. avg. of 3.21 N).

By promoting enhanced osseointegration, rhBMP-2 helps the TADs serve as stable anchors while avoiding puncture of the bone. In addition, butyl cyanoacrylate plays a key role in providing TAD stabilization until osseointegration can be achieved. As evident in the results, TADs which have become loose or migrated early in the implantation stages will have minimal or no osseointegration. With the use of TADs as stable anchors, teeth movement can be controlled without causing movement to the surrounding teeth and with enhanced bone formation at the TAD site, this eliminates the need for direct insertion through the gingival tissue and into the bone which reduces the chances of developing bone infections. These characteristics can provide a significant clinical benefit for orthodontic patients.

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Appendix A

Rat Surgery Details from Lab Notebook

Table A-1 – Rat Surgery Details

Rat #	Date	Sponge Type	Time	Action
5	10/3/2008	Control, sponge on top	8:00pm	IP injection of 0.22cc Ketamine and 0.25cc Xylazine Subcutaneous injection of 0.0495cc Buprenorphine and 15cc saline
			8:08pm	Apply 0.09cc saline on collagen sponge
			8:09pm	Start incision
			8:39pm	Surgery completed
			9:51pm	Rat movement; began recovery from anesthesia
	10/4/2008		6:20am	Subcutaneous injection of 0.0495cc Buprenorphine
	11/15/2008		5:30pm	Euthanized by CO ₂ asphyxiation
6	10/3/2008	Control, sponge on top	9:00pm	IP injection of 0.18cc Ketamine and 0.21cc Xylazine Subcutaneous injection of 0.04cc Buprenorphine and 15cc saline
			9:05pm	Apply 0.09cc saline on collagen sponge
			9:13pm	Start incision
			9:45pm	Surgery completed
			10:54pm	Rat movement; began recovery from anesthesia
	10/4/2008		6:25am	Subcutaneous injection of 0.04cc Buprenorphine
	11/15/2008		5:30pm	Euthanized by CO ₂ asphyxiation
7	10/3/2008	Control, sponge on top	9:30pm	IP injection of 0.16cc Ketamine and 0.18cc Xylazine Subcutaneous injection of 0.04cc Buprenorphine and 15cc saline
			9:45pm	Additional IP injection of 0.10cc Ketamine and 0.10cc Xylazine
			10:00pm	Additional IP injection of 0.15cc Ketamine and 0.17cc Xylazine
			10:20pm	Additional IP injection of 0.16cc Ketamine and 0.18cc Xylazine
			10:25pm	Apply 0.09cc saline on collagen

Rat #	Date	Sponge Type	Time	Action
				sponge
			10:30pm	Start incision
			10:50pm	Surgery completed
			11:45pm	Rat movement; began recovery from anesthesia
	10/4/2008		6:30am	Subcutaneous injection of 0.04cc Buprenorphine
	10/8/2008		Unknown	Rat dies a few days after surgery
9	10/4/2008	Sponge on top with rhBMP-2	7:13am	IP injection of 0.19cc Ketamine and 0.22cc Xylazine Subcutaneous injection of 0.044cc Buprenorphine and 15cc saline
			7:27am	Apply 0.09cc reconstituted BMP-2 on collagen sponge
			7:32am	Start incision
			7:45am	Surgery completed
			8:45am	Rat movement; began recovery from anesthesia
	10/5/2008		8:05am	Subcutaneous injection of 0.044cc Buprenorphine
	11/15/2008		5:45pm	Euthanized by CO ₂ asphyxiation
10	10/4/2008	Sponge on top with rhBMP-2	7:45am	Apply 0.09cc reconstituted BMP-2 on collagen sponge
			7:52am	IP injection of 0.20cc Ketamine and 0.23cc Xylazine Subcutaneous injection of 0.046cc Buprenorphine and 15cc saline
			8:02am	Start incision
			8:05am	Additional IP injection of 0.10cc Ketamine and 0.10cc Xylazine
			8:21am	Surgery completed
			9:30am	Rat movement; began recovery from anesthesia
	10/5/2008		8:08am	Subcutaneous injection of 0.044cc Buprenorphine
	11/15/2008		5:50pm	Euthanized by CO ₂ asphyxiation
11	10/4/2008	Sponge on top with rhBMP-2	8:19am	Apply 0.09cc reconstituted BMP-2 on collagen sponge
			8:28am	IP injection of 0.18cc Ketamine and 0.20cc Xylazine Subcutaneous injection of 0.041cc

Rat #	Date	Sponge Type	Time	Action
				Buprenorphine and 15cc saline
			8:36am	Start incision
			8:37am	Additional IP injection of 0.10cc Ketamine and 0.10cc Xylazine
			9:00am	Surgery completed
			10:00am	Rat movement; began recovery from anesthesia
	10/5/2008		8:10am	Subcutaneous injection of 0.044cc Buprenorphine
	11/15/2008		5:50pm	Euthanized by CO ₂ asphyxiation
19	10/4/2008	Sponge on top with rhBMP-2	9:33pm	Apply 0.09cc reconstituted BMP-2 on collagen sponge
			9:48pm	IP injection of 0.20cc Ketamine and 0.23cc Xylazine Subcutaneous injection of 0.0454cc Buprenorphine and 15cc saline
			10:04pm	Start incision
			10:22pm	Surgery completed
	10/8/2008		Unknown	Rat dies a few days after surgery
21	10/5/2008	Control, sponge on top	9:08am	IP injection of 0.16cc Ketamine and 0.19cc Xylazine Subcutaneous injection of 0.0371cc Buprenorphine and 15cc saline
			9:30am	Apply 0.09cc saline on collagen sponge
			9:32am	Start incision
			9:50am	Surgery completed
			11:10am	Rat movement; began recovery from anesthesia
	10/6/2008		8:08am	Subcutaneous injection of 0.0371cc Buprenorphine
	11/15/2008		6:30pm	Euthanized by CO ₂ asphyxiation
22	10/5/2008	Sponge on top with rhBMP-2	9:40am	IP injection of 0.20cc Ketamine and 0.22cc Xylazine Subcutaneous injection of 0.0446cc Buprenorphine and 15cc saline
			9:41am	Apply 0.09cc reconstituted BMP-2 on collagen sponge

Rat #	Date	Sponge Type	Time	Action
			9:58am	Start incision
			10:18am	Surgery completed
			11:45am	Rat movement; began recovery from anesthesia
	10/6/2008		8:10am	Subcutaneous injection of 0.0446cc Buprenorphine
	11/15/2008		6:35pm	Euthanized by CO ₂ asphyxiation
23	10/5/2008	Sponge on top with rhBMP-2	10:10am	IP injection of 0.16cc Ketamine and 0.18cc Xylazine Subcutaneous injection of 0.0364cc Buprenorphine and 15cc saline
			10:44am	Apply 0.09cc reconstituted BMP-2 on collagen sponge
			10:45am	Start incision
			10:18am	Surgery completed
			11:11am	Rat movement; began recovery from anesthesia
	10/6/2008		8:15am	Subcutaneous injection of 0.0364cc Buprenorphine
	11/15/2008		6:35pm	Euthanized by CO ₂ asphyxiation
24	2/7/2009	Control, sponge on top	3:30pm	IP injection of 0.22cc Ketamine and 0.22cc Xylazine Subcutaneous injection of 0.0446cc Buprenorphine and 15cc saline
			3:40pm	Apply 0.09cc saline on collagen sponge
			3:45pm	Start incision
			4:10pm	Surgery completed
			5:00pm	Rat movement; began recovery from anesthesia
	2/8/2009		8:20am	Subcutaneous injection of 0.0446cc Buprenorphine
	3/21/2009		2:30pm	Euthanized by CO ₂ asphyxiation
26	2/7/2009	Sponge on top with rhBMP-2	4:55pm	IP injection of 0.25cc Ketamine and 0.24cc Xylazine Subcutaneous injection of 0.0465cc Buprenorphine and 15cc saline
			5:10pm	Apply 0.09cc reconstituted BMP-2 on collagen sponge

Rat #	Date	Sponge Type	Time	Action
			4:15pm	Start incision
			4:10pm	Surgery completed
			5:20pm	Rat movement; began recovery from anesthesia
	2/8/2009		Unknown	Rat dies a day after surgery
27	2/7/2009	Sponge on top with rhBMP-2	9:50pm	IP injection of 0.25cc Ketamine and 0.24cc Xylazine Subcutaneous injection of 0.0465cc Buprenorphine and 15cc saline
			9:57pm	Apply 0.09cc reconstituted BMP-2 on collagen sponge
			10:10pm	Start incision
			10:50pm	Surgery completed
			12:30am	Rat movement; began recovery from anesthesia
	2/8/2009		8:30am	Subcutaneous injection of 0.0465cc Buprenorphine
	3/21/2009		2:30pm	Euthanized by CO ₂ asphyxiation

Appendix B

Preliminary Experiment Using One Rat

One rat was used to determine the following:

1. Effectiveness of anesthesia

A mixture of Ketamine and Xylazine (44 mg/kg body weight of Ketamine, 10 mg/kg body weight of Xylazine) should be used for inducing general anesthesia. The source used to determine these dosages is Lumb and Jones Veterinary Anesthesia and Analgesia, 4th Ed. by W. J. Tranquilla. In addition, a dose of Buprenorphine should be administered subcutaneously (0.01-0.05 mg/kg) between the shoulder blades to mitigate pain [24]. The Ketamine/Xylazine mixture provides approximately 30 minutes of effective anesthesia, and a ½ dose must be available to continue the anesthesia if the surgery takes longer than expected. The Ketamine/Xylazine and Buprenorphine mixture works well because emergence from anesthesia is smoother and it has a high factor of safety. The reason why the Ketamine/Xylazine and Buprenorphine mixture is chosen over an inhalant anesthetic, such as Halothane, is because Halothane requires that the rats wear a mask during surgery. Unfortunately, this mask would interfere with the surgical site, and therefore the Ketamine/Xylazine and Buprenorphine mixture is the better choice.

2. Instrumentation needed

Tray for BMP-2 procedures, BMP-2 / ACS (already prepared with center hole on ACS and sterilized), syringes, forceps, fine forceps, ultrafine forceps,

weight boat, bench covers, sterile gowns, scrubs, mask, rat gloves, nitrile gloves, sterile gloves, Ketamine, Xylazine, Buprenorphine, shaver, depilatory cream (Veet®), Betadine solution, Petri dish with saline, surgical drapes, scissors, scalpel, butyl cyanoacrylate / applicator, glass slab for butyl, head cap, cotton gauze and swabs (both sterile and non-sterile), disposable bag for blood and contaminated items, heating pad, recovery bin, etc.

3. Preparation / Surgical procedures

A clean, uncluttered, and sanitized work surface should be used for prep and surgery. Aseptic procedures must be followed:

Pre-operative:

- a. Sterilize instruments (using the method of heat sterilization) needed for prep and surgery: tray for rhBMP-2 procedures, forceps, fine forceps, ultrafine forceps, scissors, scalpel, butyl cyanoacrylate applicator, glass slab for butyl, surgical drapes, syringes, gloves, Petri dish with saline, cotton gauze, and cotton swabs.
- b. Bring to surgical suite: sterile instruments mentioned above as well as butyl cyanoacrylate, tupperware for weighing the rat, isolation mask and cap, sterile gowns, bench covers, weight boat, and rat in the cage.
- c. Obtain in surgical suite: Ketamine, Xylazine, Buprenorphine, scrubs, rat gloves, nitrile gloves, sterile gloves, shaver, depilatory cream (Veet®), Betadine solution, disposable bag for blood and contaminated items, heating pad, recovery bin, and non-sterile cotton swabs and gauze.

- d. In an area of the surgical suite, separate from the prep and surgery area, an assistant must wash their hands, put on sterile gloves, and do the following: 1) take a 1 cm x 1 cm absorbable collagen sponge with sterile forceps and wet it with 0.09 cc of saline (for a control TAD) or 0.09cc of reconstituted rhBMP-2 (for an rhBMP-2 treated TAD) using a syringe and a sterile tray, and 2) let it stand for at least 15 minutes before use. Meanwhile, continue with the following pre-operative procedures.
- e. Weigh the animal in a weight boat to determine the appropriate dosages of Ketamine, Xylazine, and Buprenorphine. The amount of anesthesia given to a rat is determined by the following criteria: 44 mg/kg body weight of Ketamine, 10 mg/kg body weight of Xylazine, and 0.01 – 0.05 mg/kg body weight of Buprenorphine. Record the dosages in the lab notebook.
- f. Place a bench cover down on the table.
- g. Put on rat gloves and grab the animal around the torso to administer the anesthesia. Use a mixture of Ketamine and Xylazine (44 mg/kg body weight of Ketamine, 10 mg/kg body weight of Xylazine) for inducing general anesthesia. This will provide approximately 30 minutes of effective anesthesia.
- h. In addition, administer a dose of Buprenorphine subcutaneously (0.01 – 0.05 mg/kg body weight) between the shoulder blades. The Ketamine/Xylazine and Buprenorphine mixture works well because emergence from anesthesia is smoother and has a high factor of safety.

Place the rat in a chamber until the anesthesia takes effect and the animal is down.

- i. Throw out the bench cover and place a new one for prepping. An assistant will start the prepping of the animal while the individual performing the surgery starts the surgical scrub and puts on a sterile gown to prepare for surgery.
- j. Assistant: put scrubs and nitrile gloves on for prepping procedures.
- k. Take the rat out of the chamber and place on the bench cover. Use the clippers provided in the surgical suite to shave the surgical site (rat's head). Then, apply depilatory cream on the surgical site. Wait a few minutes for the depilatory cream to set. Rub the surgical site with a damp 2 x 2 cotton sponge to remove excess hair.
- l. Pour Betadine antiseptic solution on a swab and apply it to the rat's head in a circular motion. Repeat a minimum of 3 times. Put the animal back in the chamber.
- m. Throw away the bench cover.
- n. Disinfect the table to maintain a clean, uncluttered, and sanitized area.
Use gauze sponge to wipe it down.
- o. Place down a new bench cover.
- p. Put the rat down on the new bench cover.
- q. Take off nitrile gloves and wash hands.
- r. Take pre-sterile surgical packages with surgical drape and sterile instruments, sterile gloves, isolation mask and cap, butyl

cyanoacrylate/applicator, glass slab, gauze, and cotton swabs and place them on the table within reach of the individual performing surgery.

- s. Put the heating pad in the microwave so it is ready to heat up once surgical procedures are completed.
- t. Open up the sterile surgical package cover, and open up covering for sterile instruments (without touching the actual instruments).
- u. Once the “surgeon” has scrubbed and put on the sterile gown, he/she must put on the sterile gloves and sit down to begin surgery.
- v. The surgeon should now pull out the surgical drape from the sterile surgical packages and place it on the animal. Use forceps and scissors to cut drape at incision site. Use other forceps and scissors to do actual incision (internal work).

Operative:

- a. Begin surgery with sterile instruments and handle instruments aseptically.
- b. Using the “tips-only” technique, make an incision along the line from the middle of the eyes to the middle of the ears.
- c. Once the incision is made, carefully fold back the periosteum using sterile instruments (i.e. scalpel, forceps, etc.).
- d. When the bony complex on the surface of the cranium is exposed, use sterile forceps to pick up a titanium implant, contour it to fit the skull shape, and place it on the bony complex. With the help of an assistant, glue the implant to the bone with butyl cyanoacrylate. Wait

- approximately 2 minutes to set. Use a sterile instrument to place the collagen sponge on top of the implant. The collagen sponge will be soaked in saline or rhBMP-2 depending on whether it is a control implant or not.
- e. While surgery is in progress, continually monitor the animal's vital signs.
 - f. Once the implant is stable, place as much of the periosteum over the implant.
 - g. Use sterile forceps to hold the sides of skin at the incision site together while an assistant closes the incision site using butyl cyanoacrylate. At this point, have another assistant go to the microwave to heat up the circulating heating pad for about 3-4 minutes to heat up to 37 degrees.

Post-Operative:

- a. Remove the surgical drape.
- b. Give the rat a subcutaneous injection of Buprenorphine (0.01 - 0.05 mg/kg).
- c. Move the rat to a warm area (recovery bin) to return its body temperature back to normal. In the recovery bin, place the rat on a blue bench cover, above a heating pad, to allow it to recover. Return the animal to its routine housing only after full recovery from anesthesia. Each rat will be caged separately.
- d. Buprenorphine (0.01 -0.05 mg/kg) should be administered 3 times a day for 2 days as necessary for pain management. In the event of an infection, the wound site will be cleaned 2 times/day with Betadine solution.

- e. The rats will be visually inspected daily post-op for infection. A surgical record (procedure, date, anesthesia dose, route of administration) will be maintained.

4. Surgical site to least elicit blood flow, angulations, and location of incision

The incision should be made in such a way that it elicits the least amount of blood flow. In terms of the vascular structures in a rat, the common carotid artery splits into the internal and external carotid arteries ventral to the auditory bulla (bony capsule enclosing the middle and inner ear). The internal carotid artery moves anteriorly to supply blood to the brain and the external carotid artery moves anteriorly to supply blood to most of the structures of the head. To avoid compromising the rat's vascular system, an incision approximately 1 inch in length should be made along the midline of the skull. The midline incision in the scalp should be made using firm pressure to guarantee a clean cut in a single stroke. The incision should be made from the area between the eyes to the area between the ears. Then, once the incision is made, the periosteum can be carefully removed. The periosteum can be pulled back and removed from the skull using scalpel, forceps and scissors.

5. Implant contour, adhesion method, and incision site closure

The site(s) should be chosen such that the TAD follows the contour of the skull in the best way possible. The TAD should be placed on the cranial bone such that its distance from the cranial surface is as close as possible. Only one

implant can be successfully placed on the skull and covered with the periosteum. The periosteum is too thin and does not sufficiently cover two implants at one time. Therefore, only one implant will be used for each rat. Butyl cyanoacrylate will be used to ensure the adhesion of the implant to the bone and for closure of the incision.

6. Implant size and protruding length

One 7 mm diameter TAD can fit on one rat skull. A protruding length of 2 mm on one side is sufficient enough for biomechanical testing and small enough to prevent the rat from knocking it off. The surgical procedures should be purely subcutaneous so that the protruding part of the implant will be under the skin.

Appendix C

Surgery Checklist

Date _____

Rat Skull Surgery

Initials _____

Rat Information

Breed: _____

Sex: _____

Weight: _____

Tag: _____

Cage: _____

Materials

Sterilized Instruments

- ___ 1. forceps
- ___ 2. fine forceps
- ___ 3. ultrafine forceps
- ___ 4. scissors
- ___ 5. scalpel
- ___ 6. cotton gauze
- ___ 7. cotton swabs

Bring to Surgical Suite

- ___ 8. sterile surgical drapes
- ___ 9. sterile syringes
- ___ 10. butyl cyanoacrylate/applicator/glass slab
- ___ 11. sterile gown, mask and cap
- ___ 12. bench covers
- ___ 13. weight boat

Obtained in Surgical Suite

- ___ 14. anesthesia (Ketamine and Xylazine)
- ___ 15. analgesic (Buprenorphine)
- ___ 16. scrubs
- ___ 17. rat gloves
- ___ 18. sterile gloves
- ___ 19. shaver
- ___ 20. depilatory cream (Veet®)
- ___ 21. Betadine solution
- ___ 22. disposable bag for blood and contaminated items
- ___ 23. heating pad
- ___ 24. recovery bin
- ___ 25. non-sterile cotton swabs
- ___ 26. non-sterile cotton gauze

rhBMP-2/ACS Preparation or Saline/ACS Preparation

- ___27. Take a 1 cm x 1 cm absorbable collagen sponge and wet it with 0.09cc of saline (for a control TAD) or 0.09 cc of reconstituted rhBMP-2.
- ___28. Let collagen sponge stand for at least 15 minutes before use.

Surgery Preparation

- ___29. Weigh animal in weight boat.
- ___30. Put on rat gloves and grab animal around torso to administer anesthesia. Use a mixture of Ketamine and Xylazine (44 mg/kg body weight of Ketamine, 10 mg/kg body weight of Xylazine). Record dosages in the lab notebook.
- ___31. Give the rat a subcutaneous injection of Buprenorphine (0.01 – 0.05 mg/kg body weight). Record dosage in the lab notebook.
- ___32. Place rat in chamber (anesthesia box) to let the anesthesia take effect.
- ___33. Surgeon: put on mask/cap, perform surgical scrub, and put on sterile gown and gloves to prepare for surgery.
- ___34. Assistant: put scrubs and nitrile gloves on. Perform prepping procedures below.
- ___35. Place bench cover on table for preparation procedures.
- ___36. Take rat out of chamber and place on bench cover.
- ___37. Shave hair on surgical site (rat's head) using clippers.
- ___38. Apply depilatory cream to surgical site and wait a few minutes.
- ___39. Rub surgical site with a damp 2 x 2 cotton sponge to remove excess hair.
- ___40. Apply Betadine solution on the rat's head a minimum of 3 times in a circular motion.
- ___41. Return rat to chamber.
- ___42. Throw away bench cover.
- ___43. Disinfect table. Use gauze to wipe it down.
- ___44. Place down a new bench cover.
- ___45. Put the rat down on the new bench cover.
- ___46. Take off nitrile gloves and wash hands.
- ___47. Place pre-sterile surgical packages (with sterile drape), sterile instruments, sterile gloves, and isolation mask and cap on the table.
- ___48. Place heating pad in microwave so it is ready to heat up once surgery is done.
- ___49. Put on mask and cap.
- ___50. Open up sterile surgical packages (sterile drape and instruments) without touching the inside of the sterile packages.

Surgery

- ___51. Surgeon: pull out sterile surgical drape from sterile package and put over rat. Perform surgery procedures below.
- ___52. Use forceps and scissors to cut drape at incision site.
- ___53. Make an incision along the midline of the rat's head, from the middle of the eyes to the middle of the ears.
- ___54. Carefully fold back the periosteum using forceps.

- ___55. Use sterile forceps to pick up a titanium implant and contour it to fit the shape of the skull.
- ___56. Place implant on bony complex.
- ___57. With the help of an assistant, glue implant to bone with butyl cyanoacrylate. Let it set for about 2 minutes.
- ___58. Use a sterile instrument to place the collagen sponge/rhBMP-2 or collagen sponge/saline on top of the implant.
- ___59. Close the incision using butyl cyanoacrylate. At this time, have an assistant microwave the heating pad for about 45 seconds.

Post-Surgical

- ___60. Remove surgical drape.
- ___61. Give the rat a subcutaneous injection of Buprenorphine (0.01 – 0.05 mg/kg).
- ___62. Place rat in recovery bin, on a blue bench cover, above a heating pad to allow to recover.
- ___63. Return rat to its routine housing only after full recovery from anesthesia. Each rat will be caged separately.

Appendix D

Preliminary Experiment Using INFUSE® Bone Graft Sample Kit

Note: A buffer was used to substitute for the rhBMP-2 in the sample kit

Questions to be answered from the experiment:

- Is it possible or necessary to separate the rhBMP-2 and sterile water into separate vials for smaller samples? If so, how should this be done?
- Is the effectiveness of the rhBMP-2 / sterile water mixture compromised if it is not used all at one time?
- What tools are necessary to cut the absorbable collagen sponge (ACS) into its desired shape?
- Should the collagen sponge be cut before or after the rhBMP-2 / sterile water mixture is applied to it?
- How much ACS is need for one implant?
- 4.0 mL of the rhBMP-2 / sterile water mixture is needed for 3 collagen sponges. How much of the reconstituted rhBMP-2 is needed for just 1 collagen sponge? $\frac{1}{2}$ of a collagen sponge? Etc.?
- How can a hole be created in the middle of the shaped sponge for the protruding part of the implant?
- Since the kit states that the wet collagen sponges must be used within 2 hours, how many rats can undergo surgery within 2 hours with the assumption that the sponges will be prepared before the surgeries begin?

Experimental procedure

- Take a 10 mL syringe out into the field, and draw 8.4 mL of sterile water into the syringe.
- Inject 8.4 mL of sterile water into the buffer powder vial and swirl the vial to ensure adequate mixing.
- Open the collagen sponge package and take out one of the collagen sponges.

- Since 4 mL of the rhBMP-2 / sterile water mixture is needed for 3 collagen sponges, calculate the amount of mixture needed for 1 collagen sponge: $4 \text{ mL} / 3 \text{ sponges} = 1.33 \text{ mL} / \text{sponge}$.
- Use another 10 mL syringe to withdraw 1.33 mL of reconstituted rhBMP-2 from the vial.
- Uniformly distribute 1.33 mL of reconstituted rhBMP-2 on 1 of the 2.5 cm x 5 cm collagen sponges.
- Allow the wet collagen sponge to stand for 15 minutes.
- After 15 minutes, use 2 different tools to cut the collagen sponge in the shape of the implant.
- Place a sample implant on the collagen sponge and used an x-acto knife to cut around the outer edge of the implant.
- Using another section of the collagen sponge, use a 1-hole punch to punch a hole through the wet sponge.
- Take out a second collagen sponge. This time, cut the non-wetted sponge with the x-acto knife. Likewise, use the 1-hole punch on the non-wetted sponge.

Results and Conclusions of Preliminary Experiment

- It is not necessary to separate the rhBMP-2 and sterile water into smaller vials. As long as the reconstituted rhBMP-2 is refrigerated, it can be used at another point in time. The effectiveness of the rhBMP-2 / sterile water mixture is not compromised if it is not used all at once. The kit instructions state that the wet collagen sponge must be used within 2 hours, but there is nothing stating that the rhBMP-2 / sterile water mixture must be used within 2 hours.
- The 1-hole punch is not a good tool for the purposes of this study because it punches out a diameter that is smaller than the diameter of the implant for both the non-wetted and wet sponge.
- It is difficult to cut a circular shape with the x-acto knife on a wet sponge. However, the x-acto knife works better when making straight cuts on the wet sponge.

- It is difficult to cut a circular shape through a non-wetted sponge with the x-acto knife, but easy to cut straight lines.
- Cutting the non-wetted sponge is easier than cutting the wet sponge.
- A 1.00 cm x 1.25 cm section (1/10 of 1 collagen sponge) of a non-wetted sponge is needed for 1 implant. Once the sponge is wet, it can be shaped circularly to follow the shape of the implant.
- The successful way of applying the collagen sponge to the implant:
 1. Cut one non-wetted collagen sponge (2.5 cm x 5 cm) into 10 equal sections (1.00 cm x 1.25 cm).

Figure D-1 – Collagen Sponge Dimensions

Note: Figure not drawn to scale

2. Use a plastic head pushpin to create a hole in the center of one 1.00 cm x 1.25 cm piece for the protruding part of the implant.
3. Apply the rhBMP-2 / sterile water mixture to that same 1.00 cm x 1.25 cm section. Since 4 mL of the rhBMP-2 / sterile water mixture is needed for 3 collagen sponges, the amount of mixture needed for 1 collagen sponge is $4 \text{ mL} / 3 \text{ sponges} = 1.33 \text{ mL} / \text{sponge}$. However, since one collagen sponge is divided into 10 equal sections, $1/10 * 4/3 = 0.133 \text{ mL}$ of the rhBMP-2 / sterile water mixture is needed per section. The syringe given in the kit is a 10 mL syringe with increments of 0.2 mL. For better accuracy, use a smaller syringe with smaller increments.

4. After waiting at least 15 minutes, place the wet collagen sponge over the implant and shape the collagen sponge into a circular form (which follows the shape of the implant).
- Since the wet collagen sponge must be used within 2 hours, only wet a 1.00 cm x 1.25 cm section of absorbable collagen sponge before each rat goes through surgery.
 - Note: the collagen sponge pieces used during the actual surgery were different in size than those used during the experimental phase. Specifically, 1 cm x 1 cm pieces were used with 0.09 cc of reconstituted rhBMP-2.

Appendix E

Anesthetics/Analgesics

Bottle Concentrations (mg/mL)

Ketamine HCl: 100 mg/mL

Xylazine: 20 mg/mL

Buprenorphine HCl: 0.3 mg/mL

Dosage (mg/kg of body weight)

Ketamine HCl: 44 mg/kg

Xylazine: 10 mg/kg

Buprenorphine HCl: 0.03 mg/kg

Table E-1 - Number of mL Administered of Each Drug Based on Weight

Weight of Rat (g)	Weight of Rat (kg)	Ketamine (mL)	Xylazine (mL)	Buprenorphine (mL)
200	0.200	0.088	0.100	0.020
205	0.205	0.090	0.103	0.021
210	0.210	0.092	0.105	0.021
215	0.215	0.095	0.108	0.022
220	0.220	0.097	0.110	0.022
225	0.225	0.099	0.113	0.023
230	0.230	0.101	0.115	0.023
235	0.235	0.103	0.118	0.024
240	0.240	0.106	0.120	0.024
245	0.245	0.108	0.123	0.025
250	0.250	0.110	0.125	0.025
255	0.255	0.112	0.128	0.026
260	0.260	0.114	0.130	0.026
265	0.265	0.117	0.133	0.027
270	0.270	0.119	0.135	0.027
275	0.275	0.121	0.138	0.028
280	0.280	0.123	0.140	0.028
285	0.285	0.125	0.143	0.029
290	0.290	0.128	0.145	0.029
295	0.295	0.130	0.148	0.030
300	0.300	0.132	0.150	0.030
305	0.305	0.134	0.153	0.031
310	0.310	0.136	0.155	0.031

315	0.315	0.139	0.158	0.032
320	0.320	0.141	0.160	0.032
325	0.325	0.143	0.163	0.033
330	0.330	0.145	0.165	0.033
335	0.335	0.147	0.168	0.034
340	0.340	0.150	0.170	0.034

Appendix F

Butyl Cyanoacrylate and the Effect of UV Light on Curing Time

In the presence of moisture, cyanoacrylates rapidly polymerize and set quickly. A quick and easy way to test whether a certain type of cyanoacrylate will adhere to tissue with degrees of moisture is to apply the cyanoacrylate to designated sections of the implant and see if it adheres to cow bone or fresh meat from the grocery store. The tissue found in cow bone is similar to the tissue found in rats, and therefore using the cow bone allows for quick and effective testing without having to perform surgery on the rat for this preliminary step. In addition, saliva added to the site of the implant accounts for the presence of blood during the actual surgery. This aids in determining whether the saliva, with chemical and physical components somewhat similar to blood, will have any effect on the implant's ability to adhere. From this experiment, the curing time and whether the cyanoacrylate is an effective adhesive can be determined. Moreover, the effect of UV light (which has been known to reduce curing time) will also be evaluated. Finding a way to reduce the curing time is desired to ensure implant stability and to minimize the amount of blood flow and other fluids into the implant site.

An experiment using Vetbond butyl cyanoacrylate was performed. The results from the experiment can be found in the following table:

Table F-1 – Vetbond Butyl Cyanoacrylate Curing Time

All trials used saliva to account for the presence of blood				
	Curing Time Without UV Light (Trial 1)	Curing Time Without UV Light (Trial 2)	Curing Time With UV Light (Trial 1)	Curing Time With UV Light (Trial 2)
Cow Bone	2 minutes	2 minutes	1 minute, 50 seconds	1 minute, 55 seconds
New York Steak Bone In (Shaved Bone)	2 minutes	2 minutes	1 minute, 50 seconds	2 minutes

The curing time was determined based on the following graphs:

Figure F-1 – Curing Time Using Cow Bone without UV Light

Figure F-2 – Curing Time Using NY Steak without UV Light

Figure F-3 – Curing Time Using Cow Bone with UV Light

Figure F-4 – Curing Time Using NY Steak with UV Light

Several conclusions were made from the above experiment:

- Butyl cyanoacrylate is an effective adhesive.
- Saliva does not affect the ability of the implant to adhere to bone. Thus, blood, with chemical and physical components somewhat similar to blood, is also assumed to have little effect on the ability of the implant to adhere to bone.
- The curing time with UV light is less than the curing time without UV light.

However, the difference is not that significant.

Appendix G

Bone Coverage Raw Data

Table G-1 – Photographs of TAD Bone Coverage for Each Rat

Rat #	Control or rhBMP-2 treated	Bone Coverage (Photo taken prior to biomechanical testing)
Rat 5	Control, sponge on top
Rat 6	Control, sponge on top	No bone coverage observed – TAD migrated
Rat 7	Control, sponge on top	N/A - rat died after surgery
Rat 9	Sponge on top w/ rhBMP-2
Rat 10	Sponge on top w/ rhBMP-2

Rat 11	Sponge on top w/ rhBMP-2
Rat 19	Sponge on top w/ rhBMP-2	N/A– rat died a few days after surgery		
Rat 21	Control, sponge on top
Rat 22	Sponge on top w/ rhBMP-2
Rat 23	Sponge on top w/ rhBMP-2

Rat 24	Control, sponge on top
Rat 26	Sponge on top w/ rhBMP-2	N/A - Rat died one day after surgery		
Rat 27	Sponge on top w/ rhBMP-2

Appendix H

Biomechanical Testing Failure Load Graphs

Note: No graphs are available for Rats 5, 6, 7, 19, 26. See explanation in the Results

Summary table in Section 4.2.1.

Figure H-1: Failure Load for Rat #9

Figure H-2: Failure Load for Rat #10

Figure H-3: Failure Load for Rat #11

Figure H-4: Failure Load for Rat #21

Figure H-5: Failure Load for Rat #22

Figure H-6: Failure Load for Rat #23

Figure H-7: Failure Load for Rat #24

Figure H-8: Failure Load for Rat #27