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# Using deep learning to assess new bone formation after bone grafting

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BOSTON UNIVERSITY  
HENRY M. GOLDMAN SCHOOL OF DENTAL MEDICINE

THESIS

**USING DEEP LEARNING TO ASSESS NEW BONE FORMATION AFTER  
BONE GRAFTING**

by

**ELIAS A. EXARCHOS**

B.S. Biomedical Engineering, Boston University, 2015  
D.M.D., Boston University, 2019

Submitted in partial fulfillment of the requirements for the degree of

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2022

Approved by:

First Reader

---

Taisuke Ohira, DDS, PhD  
Clinical Assistant Professor, Department of Periodontology

Second Reader

---

Wayne A. Gonnerman, PhD  
Assistant Professor, Department of Periodontology

Third Reader

---

Serge Dibart, DMD  
Chair, Department of Periodontology

## **DEDICATION**

I would like to dedicate this work to my patient wife, my loving mother who has always supported me, and my father who has always inspired me and encouraged me to never give up, no matter what.

## **ACKNOWLEDGMENTS**

I would like to express my deepest appreciation to my mentors Dr. Taisuke Ohira and Dr. Serge Dibart, for their continuous help and willingness to teach and inspire. Without their constant support and passion, this research would not have been possible.

USING DEEP LEARNING TO ASSESS NEW BONE FORMATION AFTER BONE  
GRAFTING

ELIAS A. EXARCHOS

Boston University, Henry M. Goldman School of Dental Medicine, 2022

Major Professor: Taisuke Ohira, DDS, PhD

Clinical Assistant Professor of Periodontology

ABSTRACT

**Background:** The ultrasonic vibrations from the piezoelectric knife may amplify the natural response to surgical injury. This may lead to different clinical and biological outcomes when using the piezoelectric knife versus a surgical bur to create selective cortical penetrations for alveolar ridge augmentation surgeries. The first aim of this study was to analyze the differences in bone graft healing when selective cortical penetrations are created with a surgical bur and with a piezoelectric knife. The second aim of this pilot study was to see if enhanced new bone formation during bone regeneration procedures can be achieved with the use of a piezoelectric knife versus the conventional bur or onlay grafting techniques utilizing deep learning, a subset of machine learning.

**Materials & Methods:** The project was approved by the Boston University Medical Center Institutional Animal Care and Use Committee (IACUC). Twenty, 9-10 week male Sprague Dawley rats, weighing approximately 300g, were used in this study. The rats

were randomly divided into three groups: *Xenograft*, *Alloplast*, and *Collagen*. These groups were further divided by surgical technique: *Bur*, *Piezo*, and *Onlay*. For the Bur and Piezo groups, four equally-spaced selective cortical penetrations were made prior to bone graft stabilization. Three rats served as controls (*Control* group). Microcomputed tomography scans ( $\mu CT$ ) were acquired for each sample, containing approximately 1,000 slices of data each. After 28 days of healing the volumes of and density of the newly formed bone were extracted and analyzed for each group. This was achieved with an innovative deep learning algorithm designed for multi-level segmentation and regional feature detection utilizing convolutional neural networks (*CNN*).

**Results:** Microcomputed tomography ( $\mu CT$ ) of our samples yielded very localized, high-resolution scans of our surgical samples. The innovative deep learning algorithm was able to reliably produce highly accurate, unbiased segmentations of our samples. This study demonstrated that new bone formation was possible with all nine of the tested surgical techniques, however the differences were not statistically significant. Selective cortical penetrations with a piezoelectric knife (*PIEZO*) resulted in significantly more “cortical-like” new bone formation at 28 days.

**Conclusion:** Within the limitations of this preliminary study, it is possible to conclude that the piezoelectric knife is a valid alternative to conventional carbide burs when making selective cortical penetrations prior to bone grafting surgery. Additionally, our deep learning algorithm successfully segmented thousands of slices of data and allowed for the calculation of porosity and new bone volume in our samples.

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## LIST OF ABBREVIATIONS

|              |   |
|--------------|---|
| $\mu$ CT     | Micro-Computed Tomography   |
| ADO          | Alveolar Distraction Osteogenesis                                       |
| AI           | Artificial Intelligence   |
| BMU          | Basic Multicellular Unit  |
| BU           | Boston University   |
| FOV          | Field of View   |
| GBR          | Guided Bone Regeneration  |
| ISO          | International Standards Organization                                    |
| MSC          | Mesenchymal Stem Cell   |
| RANK-L       | Receptor Activator or Nuclear Factor kappa-B Ligand                     |
| RAP          | Regional Acceleratory Phenomenon  |
| rhPDGF-BB    | recombinant human Platelet-Derived Growth Factor-BB                     |
| ROI          | Region of Interest  |
| SCP          | Selective Cortical Penetrations   |
| SMART        | Subperiosteal Minimally Invasive Aesthetic Ridge Augmentation Technique |
| TGF- $\beta$ | Transforming Growth Factor $\beta$                                      |

## **CHAPTER 1**

### **INTRODUCTION**

#### **1.1 Bone Grafting in Periodontology**

Advancements in the field of implant dentistry have given rise to various materials and methods designed to avoid alveolar ridge augmentation. However, clinicians still often encounter situations where inadequate maxillary or mandibular bone volume inhibits the esthetic or functional placement of dental implants. These alveolar bone deficiencies can be caused by periodontal disease, alveolar resorption subsequent to tooth loss, trauma, long-term use of a removable prosthesis, pathology, or congenital defects. [1] [2] [3]

Adequate volume of alveolar bone, the main requirement for successful long-term dental implants, can be restored in the edentulous alveolar ridge through various surgical procedures. Several ridge augmentation techniques, e.g. guided bone regeneration (GBR), ridge splitting and expansion, and autologous bone block grafting, have been tested extensively and shown to predictably augment bone height and width. [4] Although these procedures are usually successful, each hard tissue augmentation procedure has advantages and disadvantages. And, each patient's treatment plan should be tailored based on a fundamental understanding of the literature, both current and classic, and the biology and anatomy of dentoalveolar bone.

## 1.2 Types of Bone Grafts

Bone grafts used in dentistry develop bone through three possible mechanisms: direct osteogenesis, osteoconduction, and osteoinduction. Grafts may regenerate bone from one, two, or all three of these mechanisms to varying degrees. [5]

- A. *Osteoconductive*: Graft material does not directly contribute to new bone formation but serves as a scaffold for bone formation by adjacent host bone.
- B. *Osteoinductive*: Bone formation is induced in the surrounding soft tissue immediately adjacent to the grafted material.
- C. *Osteogenic*: New bone formation occurs as a result of bone-forming cells contained in the graft.

Osteoconduction supports the attachment of osteoblasts and osteo-progenitor cells and to allow the migration and ingrowth of these cells within the three-dimensional architecture of the graft. [6] Osteoinduction means that the graft can induce the primitive, undifferentiated and pluripotent cells to develop into the bone-forming cell-lineage, by which osteogenesis is induced. [7] Osteogenesis allows osteodifferentiation and subsequent new bone formation occurs through donor cells derived from either the host or the grafts. [8] [9] Different bone grafting materials have been used in dentistry with the purpose of replacing/grafting of human bone, but each product/material has its own particular advantages and disadvantages, and they are classified as being either *autografts*, *allografts*, *xenografts*, or *alloplasts*. [10]

### 1.2.1 Autografts

Autogenous grafts are transferred from one position to another within the same individual. In periodontology and oral surgery, this means that a second surgical site is needed (e.g., retromolar pad, maxillary tuberosity). Autogenous grafts are also known as autografts and are the gold standard to which all other grafting materials are compared because they possess all the previously mentioned healing mechanisms (osteogenicity, osteoinductivity, and osteoconductivity). In addition, because they are from the same individual, they obviate graft-host reactions mediated by histocompatibility mismatches because of the absence of antigenicity. [11]

For the purpose of this animal study, autografts were not part of the experimental model because of the difficult and complex surgery required to harvest such grafts.

### 1.2.2 Allografts

Allografts, also called allogenic, homologous, or homografts, are composed of materials taken from genetically dissimilar members of the *same species*. Allografts are histocompatible and are available in various forms, including demineralized bone matrices, cancellous chips, cortico-cancellous grafts, cortical grafts, and osteochondral or whole-bone segments, depending on the host's requirements. Although viable cells are lacking, allogenic bone grafts provide similar mechanical properties as the autologous

bone grafts, and it may also contain the collagenous matrix/network and proteins of natural bone. [12]

Since this study was conducted on Sprague-Dawley rats, allografts were not included in the experimental model simply because there are no commercially available bone grafts derived from this species.

### *1.2.3 Xenografts*

Xenografts, also known as heterografts or xenogenic grafts, are from donors of another species. Several studies have shown successful hard tissue regenerative outcomes with xenogenic grafts and they hold a major role in dental, periodontal, and cranio-maxillofacial applications. In randomized controlled clinical trials [13] [14] a bovine xenograft or a synthetic bone substitute both presented similar radiographic alveolar bone changes when used for alveolar ridge preservation procedures or ridge augmentation procedures adjacent to dental implant implants. [15]

A xenogenic bone graft (Bio-Oss Collagen) was used for this animal study (*Xenograft* study group). Bio-Oss Collagen is a mixture of spongiosa (cancellous) bovine bone granulate (xenograft) and porcine collagen fibers. The material is 10% Type I collagen by volume. It is an allergen-free bone substitute and several histologic and histomorphometric studies have shown that it can successfully form lamellar, parallel-

fiber, and woven bone. [16] This bovine xenograft seems to resorb very slowly in humans compared to other bone graft materials and residual particles are usually present for long periods of time in close/intimate contact with the newly formed native bone and osteoid tissue.

#### *1.2.4 Alloplasts*

Alloplasts, also known as alloplastic or synthetic grafts, are artificial or manufactured materials used as a substitute for natural bone graft materials and can be subdivided based on their origin and chemical composition. Alloplastic bone graft materials include calcium sulfates, hydroxyapatites, tricalcium phosphates, calcium phosphate cements/ceramics, and bioactive glass. [17] Although they lack osteoinductive or osteogenic properties, synthetic bone substitutes are in increasing demand as the aging population increases. Moreover, they have demonstrated the promising potential to be biomaterials for bone tissue engineering due to their controllable and tunable biomechanical and biodegradability properties. [18] Also, they provide better controllability in terms of porosity, physiochemical structure and immunologic adverse effects when compared to other types of bone grafting materials. [19] [20]

For this study, in addition to a xenograft study group, a non-sintered, resorbable calcium apatite-based *Alloplast* bone graft material (OsteoGen) was used. These calcium apatite crystals are embedded in Type I bovine Achilles tendon cartilage. The final

product is easy to handle as it comes pre-made in a pliable sheet that simplifies the preparation, application, and delivery of the graft material (OsteoGen Strips). These “strips” address the clinical need for a synthetic bone substitute and have been used extensively in extraction socket grafting and immediate implant-related bone grafting procedures. It was approved for use by the FDA in 2009 [21] and what is unique about this material is the low temperature preparation technique. This non-sintered product uses a low-temperature manufacturing process (so as to not distort the physical form of the crystal) that generates osteoconductive and resorbable low-density crystals and crystal clusters with distinctive calcium to phosphate ratios that makes it neither a beta-tricalcium phosphate nor a dense non-resorbable ceramic hydroxyapatite. [22] [23]

#### *1.2.5 Other*

A third “bone grafting material” was tested in this study and it is not an autograft, an allograft, a xenograft, nor an allograft. It is not classified as a “bone graft”; it is a collagen matrix (*Collagen* study group). Geistlich’s Mucograft provides short-term volume-stability and cell-occlusive properties. And we tested whether or not this could maintain the subperiosteal space long enough for new bone formation to occur when used in conjunction with selective cortical penetrations (SCPs). [24]

Mucograft is a resorbable porcine collagen matrix with two structures: a compact layer for tissue adherence (for favorable wound healing) and a thick spongy

layer/scaffold which facilitates and promotes formation of new blood vessels, a key requirement for soft/hard tissue regeneration healing. Mucograft has mostly been tested as a barrier membrane or alongside soft tissue augmentation procedures. It has been shown to result in minimal inflammation, an absence of multinucleated giant cells, and favorable tissue reactivity profiles.[25] It is an easy-to-handle material and can be purchased in matrices of varying sizes and shapes. Per the manufacturer, there is no need for preoperative hydration, which can also reduce chairside time. [26] [27]

### **1.3 Surgical Techniques**

GBR is not the only hard tissue augmentation method used in periodontal and oral surgery. Several studies have demonstrated successful new bone formation with other techniques as well such as: distraction osteogenesis, block grafting, ridge splitting, maxillary sinus lifting, and onlay grafting without the use of a cell-occlusive membrane.

#### *1.3.1 GBR*

Guided bone regeneration (GBR) is a surgical procedure that has been used for several years to augment alveolar bone height and/or width, in preparation for dental implant placement. This surgical procedure involves: (1) reflecting a full-thickness flap, (2) placing a bone graft against the underlying bony defect, and (3) fixing a resorbable or non-resorbable membrane over the bone graft. This thereby creates and maintains a space

under the periosteum for new bone formation to occur, while preventing non-osteogenic cells from interfering with the desired new bone growth. [3]

Ideally, for new bone regeneration, osteoprogenitor cells should colonize this space, however these cells grow slowly. In order to give the osteoprogenitor cells the ability to colonize this space, the cell-occlusive membrane used in GBR prevents the ingrowth of the rapidly proliferating epithelial and connective tissue cells into the defect. [4] This careful inclusion and exclusion of specific cells inside and away from the graft space is one of the fundamental reasons why clinicians have had very high long-term success rates with guided bone regeneration.

### *1.3.2 Distraction Osteogenesis*

Alveolar distraction osteogenesis (ADO) is a bone regeneration technique based on a biological process used for regenerating and consolidating bone between two bone segments obtained after initial osteotomy. [28] These segments are gradually separated by the process of distraction and this technique (device) can achieve increased new bone volume in both horizontal and vertical dimensions. [29] [30]

### *1.3.3 Ridge Splitting and Expansion*

Ridge splitting is a technique used to augment the alveolar ridge prior to (or simultaneously with) dental implant placement. This technique involves cutting the

cortical plate(s) deep enough into the marrow space and creating a “hinge”-like attached plate of bone that can be split and spread from the opposing wall, without being fully removed. Due to the need to enter marrow space, this technique requires special attention to the density of the bone, the distance to neighboring teeth/vital structures, and sufficient width of the original bone itself. An inlay graft (either particulate or block) is usually “sandwiched” between the two plates of bone in order to maintain the space. Several ridge split techniques have been developed in the past few decades and they include, but are not limited to, the split crest osteotomy [31] [32] and the ridge expansion osteotomy. [33]

#### *1.3.4 Maxillary Sinus Lift*

Maxillary sinus lifts are commonly used in periodontology and oral surgery to augment the hard tissue volume prior to implant placement in the posterior maxillary region(s). It involves separating the Schneiderian membrane from the internal walls of the maxillary sinuses and condensing a volume-stable grafting material underneath the periosteal space(s), allowing time for new bone formation to occur. The conventional method for maxillary sinus elevation (“direct sinus lifts”) requires surgical access through the lateral wall of the maxilla, followed by elevation of the sinus membrane and insertion of bone graft under *direct* vision. [34] [35] A modified less invasive method uses a crestal approach *without direct* vision of the Schneiderian membrane (“indirect sinus lift”). [36] These techniques do not usually require the use of a cell-occlusive membrane as long as

the Schneiderian membrane is not ruptured, and the overlaying periosteum is remained intact.

Variations of this type of technique have shown potential for new bone formation even without a bone graft in the maxillary sinus. This has been described in a variety of studies, including radiological and histological studies, and the recent studies on this topic indicate high success rates of maxillary sinus lifts without a bone graft material. A literature review by Pinchasov *et al.* concluded that the evidence clearly shows that the maxillary sinus has the potential to heal and form new bone without bone grafts or substitutes. [37] A recent meta-analysis performed by Yan *et al.* demonstrated that the available evidence suggests that predictable results can be acquired through transalveolar sinus floor lift without bone grafting, however there may be a trend towards more new bone formation with the use of bone grafts. [38]

#### *1.3.5 Subperiosteal Onlay Grafts*

Onlay grafting (without the use of a membrane) is a hard tissue regeneration method that utilizes particulate bone grafting material underneath an *intact* periosteal surface. If the periosteum is carefully maintained intact during full-thickness flap elevation, this can serve as a barrier to the migration of epithelial and soft tissue connective tissue cells, allowing new bone formation to ensue. [39] Plus, the minimally invasive nature of this method (without disrupting the soft tissues overlying the recipient

bed) results in minimal surgical trauma, postoperative discomfort, and morbidity. A number of variations of subperiosteal tunneling have been reported and they have shown successful ridge augmentation volume gains with the use of particulate hydroxyapatite and human mineralized bone allograft. [40] [41] Selective cortical penetrations of the recipient site(s), also known as “decortication”, have been recommended in conjunction with anorganic bovine bone particulate grafts when hard tissue regeneration is desired beneath subperiosteal tunnels/pouches. [42]

Dibart *et al.* reported on the use of subperiosteal tunneling with bone grafting and piezosurgical corticotomies as an adjunct to surgically facilitated orthodontic therapy. [43] Nevins *et al.* compared and reported variable outcomes with the use of recombinant human platelet-derived growth factor BB (rhPDGF-BB) in combination with three different particulate bone matrices (freeze-dried bone allograft (FDBA), anorganic bovine bone graft (ABBG), or anorganic bovine bone graft/mineralized collagen bone substitute (ABBG/MCBS)) for the treatment of maxillary anterior edentulous spaces. [44]

The MIHRA technique is a similar technique that uses a small incision and subperiosteal tunneling (Minimal Invasive Horizontal Ridge Augmentation) and has been tested with various bone graft materials (autograft, allograft, and xenograft). [45] Significant bone gain has been reported and grafting underneath an intact periosteal pouch has proven to be a valid, safe, simple, and effective method of reconstructing hard tissue defects.

Recently, Dr. Ernesto Lee has published and trademarked the “SMART” method (Subperiosteal Minimally Invasive Aesthetic Ridge Augmentation Technique). His technique involves making one or more small access incisions, reflecting a full-thickness subperiosteal pouch, and placing xenograft (bovine) particles mixed with GEM21S (rhPDGF-BB) onto the recipient bone without the use of SCPs (selective cortical penetrations). Histological and radiographic analyses have demonstrated that the SMART technique is a predictable, valid, and safe alternative to GBR procedures. [46] While this bone grafting technique is mostly used for lateral alveolar bone augmentation, it can be sequentially combined with orthodontic forced eruption to result in both horizontal and vertical new bone formation. [47]

Autogenous bone grafts are still considered the gold standard for predictable hard tissue volume gain. However, for subperiosteal onlay grafting, allografts, xenografts, and even alloplasts, such as various calcium phosphates, have been proven to be a valuable option for these types of bone augmentation procedures. [48] For our animal study, one allograft material and one xenograft material were chosen.

### *1.3.7 Bone Bioreactor*

In addition to the *Allograft* group and the *Xenograft* group, a *Collagen* group was used in this study. New bone formation in an animal model has occurred under full-thickness periosteal flaps, providing that the space is *maintained* for the necessary

amount of time for the host to form new bone under the subperiosteal space. Significant hard tissue augmentation occurs with collagen matrices and even with no grafting material, provided that the reflected, intact periosteum provides space for new bony growth. [49]

In 2005, Stevens *et al.* used an animal model to demonstrate that simply by creating and manipulating an artificial space (bioreactor) between the bone and the periosteum (a mesenchymal layer rich in pluripotent cells), the body's healing mechanism is leveraged to engineer bone that is biomechanically identical to native bone. Their group used a cross-linked calcium-alginate gel placed under a subperiosteal pouch in a rabbit tibia and found that the creation of this "*in vivo* bone bioreactor" can predictably lead to new woven bone matrix formation which matures into fully mineralized compact bone (exhibiting all the histological markers and mechanical properties of native bone). [24]

### *1.3.8 Selective Cortical Penetrations*

As previously mentioned, clinicians will often decorticate the bone prior to placing the bone graft material, with the goal of including more osteoprogenitor cells and increasing blood supply from the marrow to the space to the graft space. However, there is controversy in the literature regarding the effectiveness of selective cortical penetrations on bone grafting outcomes because its ability to accelerate or increase bone

regeneration has not been substantiated in *human* clinical trials. In 2009, Greenstein *et al* did a literature review and found that there is inconsistent literature, conflicting information, and not enough clinical trials to make a definitive determination as to the merits of bone decortication prior to GBR procedures. [2] In 2008, Adeyemo *et al* found no difference in terms of healing and integration of the bone graft between perforated and nonperforated recipient cortical beds. [3]

Rompen *et al.*, however, demonstrated greater regeneration in skulls perforated with nine 0.8 mm diameter holes (172.8%) compared with skulls with an intact osseous surface (141%). It was stated that *de novo* bone formation occurs by stimulation of the blood supply and access of bone-forming cells by cortical perforations. [50] In 2017, Danesh-Sani *et al.* conducted a human study and found that, after 7 months of healing, cortical bone perforations favorably affects the amount of new bone formation, however these results were not statistically significant. They also found that cortical bone perforations significantly increased the number of new vessels (angiogenesis) of the regenerated bone. [1]

In 2018, Wessing *et al* conducted a systematic review and meta-analysis and found that decortication in GBR procedures led to an increase in alveolar ridge width, even though those values did not reach statistical significance. [51] By and large, the research generally shows better, not worse, outcomes when utilizing selective cortical penetration techniques, as the marrow space can provide greater osteoprogenitor cell

access, better blood supply, and nourishment, especially in dense mandibular bone, however these studies are not conclusive, and clinicians can choose to decorticate at their own discretion. Further human clinical trials are required to elucidate the biologically complex effects of selective cortical penetrations on bone regeneration.

When creating selective decortications in the alveolar bone, most practitioners use a conventional surgical bur with saline irrigation, however in the past, scalpels and chisels have been used. These forms of bony injuries induce something called the regional acceleratory phenomenon (RAP), a term coined in 1981 by an orthopedic surgeon, Dr. Harold M. Frost. [52] The regional acceleratory phenomenon is characterized by an increase in vascular perfusion and bone turnover as well as a decrease in bone density (transient osteopenia). This brief phase of osteopenia is quickly followed by rapid osteoblastic activity and bone remodeling, resulting in full reestablishment of bone density. Others have described the RAP as a temporary state of accelerated healing at the site of bony injury in which there is a localized increase in cellular activity and bone remodeling through the recruitment of osteoblasts and osteoclasts. [53] This type of healing following alveolar bone decortication is bimodal in nature and is thought to consist of two interrelated, overlapping phases – a demineralization phase (early phase) and a remineralization phase (later phase). [54]

Any type of bony injury of sufficient magnitude can elicit the regional acceleratory phenomenon. In the orthodontic literature, it has been shown that selective

cortical penetrations created by surgical burs, piezoelectric knives, scalpel and mallets are all able to trigger the RAP phenomenon in surgically assisted rapid orthodontic procedures. [55] [56] [57] [58] [59] Even full-thickness mucoperiosteal flap reflection alone (a type of bony injury) can initiate RAP, although the extent of this phenomenon varies depending on the intensity of the stimulus.

Of particular interest is minimally invasive technique called “Piezocision” for surgically assisted orthodontics. This technique, as mentioned previously, was introduced by Dibart *et al.* in 2009. A piezoelectric knife is used to create selective cortical penetrations in the alveolar bone to induce the regional acceleratory phenomenon for accelerated orthodontic movement. This technique involves vertical incisions that are made in the gingiva, which can also be used advantageously for tunneling procedures in which hard tissue grafts can be placed under a subperiosteal tunnel to correct bone deficiencies. [43] And, the SCPs created by the piezoelectric knife, in theory, can accelerate the bone graft healing.

Piezocision demonstrates that decortications of the alveolar bone can be made with an ultrasonic piezoelectric knife and concurrent alveolar ridge augmentations can be completed, however today, clinicians still are largely using surgical burs to create the corticotomies in alveolar bone prior to bone grafting. But why? Horton *et al* showed that osteoblastic activity and rates of healings were increased in surgical defects of alveolar bone created by ultrasonic instruments compared to a conventional rotary bur. [60] In

2013, Canullo *et al.* showed that implant stability develops faster when the implant site osteotomy is performed with a mixed drilling/piezoelectric technique versus a conventional drilling technique. [60] And, in 2018, Fugito *et al.* demonstrated that the use of either piezoelectric tips or rotary burs generates a temperature that does not affect the healing hard/soft tissues. [62] Lastly, in 2019, Alikhani *et al* demonstrated that locally applied, high frequency vibrations, similar to those used in piezoelectric instruments, stimulates alveolar bone formation under physiologic conditions and that these locally applied vibrations can generate gradients of increased anabolic metabolism and decreased catabolic metabolism in alveolar bone. [63]

Therefore, it is conceivable that the choice to utilize a conventional surgical carbide bur or a piezoelectric knife and the choice to create selective cortical penetrations whatsoever in conjunction with bone regeneration procedures may lead to quantitative and qualitative differences in new bone formation due to the differences in tissue damage and tissue response. So, for our experiment, we further subdivided our study groups into a *Bur* group, a *Piezo* group, and an *Onlay* group with no SCPs.

#### **1.4 Biological Background**

With regards to augmenting missing hard tissue in the oral cavity or craniofacial region, basic knowledge of the bone anatomy is crucial in order to appreciate the particulars of various bone augmentation techniques. The maxilla and mandible, like most bones of the skull, are formed embryologically through intramembranous

ossification. [64] Developing alveolar bone becomes a highly mineralized tissue and, like bone in other parts of the human skeleton, has a matrix consisting of organic matter (mostly type I collagen and other proteins), inorganic matter (calcium phosphate in the form of hydroxyapatite crystals), and cells. [5] There is a dynamic relationship between these hard tissues and the cells, vasculature, and soft tissues that surround the mandibular and maxillary bone.

#### *1.4.2 Periosteum*

The outermost layer of the bone comprises the periosteum. The periosteum is a dense layered membrane responsible for partial blood supply to bone and bone fracture repair. In fact, 18th century surgeons discovered that the periosteum is capable of inducing new bone formation. [65] The first layer of the periosteum is an outer fibrous layer providing physical and structural support. The inner cambium layer contains many osteoprogenitor cells. However, the thickness of the cambium osteoprogenitor layer decreases with age, concomitant with a decrease in its osteogenic potential. [66] In some bones, there is also an endosteum. This endosteal membrane is derived from the periosteum however is very thin, averaging only 10-40  $\mu\text{m}$  in thickness, consisting of an indistinct connective tissue layer and a few layers of cells. It is thought to have similar functions to the periosteum, contributing to bone repair and reconstruction, as it houses osteoprogenitor cells such as MSCs and preosteoblasts. [67]

#### 1.4.2 Alveolar Bone

Maxillary and mandibular bone is composed of two major types: the external cortical bone and the internal cancellous bone. The outer cortical bone is found on the buccal, lingual, and crestal surfaces of alveolar ridges. This cortical bone is very dense, compact, and has very few blood vessels, especially in the mandibular jaw. Within the cortical bone is the inner cancellous bone. This trabecular, or cancellous, bone runs inside the compact bone and is generally surrounded by a marrow that is highly cellular and unlike the cortical bone, has a rich blood supply. [64] Osseous tissues of cortical bone and cancellous bone are biochemically identical, but structurally distinct.

One way the alveolar bone is classified is based on these structural differences. For instance, in 1988, Misch *et al.* proposed four major bone density groups based on macroscopic cortical and trabecular characteristics: *D1* bone was described as dense cortical bone, typically found in the anterior mandible. *D2* bone was described as porous cortical and coarse trabecular bone, often found in the anterior and posterior mandible and anterior maxilla. *D3* bone types had a thinner (porous) cortical layer with fine trabecular regions. This bone is anatomically found in the anterior maxilla and posterior maxilla and the posterior mandible. Lastly, *D4* bone was described as fine trabecular bone, typically located in the posterior maxilla. [68] A very soft bone, with incomplete mineralization and large intratrabecular spaces has been referred to as *D5* bone. This bone type is often found in the immature bone of a developing bone graft site.

The osseous tissue consists of an organic phase of mainly collagen fibers, which function to give strength, flexibility, and resistance to torsional force, and an inorganic phase of mainly hydroxyapatite crystals, which function to give the bone resistance to compression [69] Inner bone marrow tissue is considered a “blood organ”. It possesses important stem cells that are important for bone regeneration. It is a niche for hematopoietic stem cells, which can regenerate whole blood systems from as little as one cell.

#### *1.4.3 Cellular Biology*

In bone, there is a balance between bone-formation and bone resorption that is modified throughout life. Regulation is through various bone forming cells (MSCs, pre-osteoblasts, mature osteoblasts, bone-lining cells, and osteocytes) and bone resorbing cells (osteoclasts, macrophages, and multinucleated giant cells, all derived from bone marrow hematopoietic stem cells). [69] More recently, researchers discovered another cell type found in bone tissue, the osteomac, which is thought to be important in bone remodeling and homeostasis. [70]

Osteoblasts and osteoclasts are the two cells that are known to be responsible for both growth, development, deposition, and remodeling of bone. Osteoclasts are responsible for the resorption of bone. They are multinucleated cells that derive from hematopoietic progenitors in the bone marrow which also give rise to monocytes in

peripheral blood. [71] Osteoblasts, on the other hand, are known to lay down new bone and are derived from local osteoprogenitor cells. These osteoprogenitor cells, or preosteoblasts, originate from mesenchymal stem cells within the bone marrow. Remodeling of both cortical and cancellous bone involves complex interactions between osteocytes and these two cell types.

A subpopulation of osteoblasts become embedded by mineralized bone and become a different cell type, osteocytes, which form a network of membrane processes that extend throughout mineralized bone. Osteocytes also play a role in bone homeostasis as they are a major source of molecules that regulate the activity of osteoblasts and osteoclasts, such as RANKL and sclerostin. It is believed that they respond to mechanical load, initiate, and direct the remodeling process that repairs damaged bone. [72] Apoptosis of osteocytes is one of most accepted signaling events for targeted bone remodeling, a process whose goal is to renew and maintain bone throughout life. [64] The concept of osteocyte apoptosis inducing bone remodeling was first introduced in the 1960s by Dr. Harold Frost. While studying the regional acceleratory phenomenon (RAP), he suggested that *microdamage* is needed to for the bone to be actively remodeled in order to adapt to a changing mechanical environment.

The osteocyte normally secretes transforming growth factor  $\beta$  (TGF- $\beta$ ), which inhibits osteoclastogenesis. When osteocyte apoptosis occurs in response to mechanical trauma, such as piezoelectric stimulus for example, TGF-  $\beta$  levels are lowered, which

removes these inhibitory signals and allows osteoclast formation, one of the key initiators of bone remodeling. [73] The osteocyte response to various surgical stimuli and their connections to the bone matrix and the bone remodeling process are believed to be key drivers in bone and bone graft healing.

#### *1.4.4 Bone Healing*

A fundamental knowledge of bone healing is necessary to understand the complexities of various bone grafting techniques. After initial stimulus/injury bone will heal either by direct intramembranous or indirect fracture healing, which consists of both intramembranous and endochondral bone formation. Frost *et al.* divided the bone response to injury/fracture stimulation into five stages. [54]

- A. Bone intervention (e.g., fracture, injury)
- B. Granulation tissue (temporary healing involving soft tissues)
- C. Callus formation
- D. Lamellar bone formation
- E. Recontouring/bone modeling

Our body's biological response to any tissue injury begins with an inflammatory response. The sites of stimulus send downstream signals which recruit various immune and host defense cells. [73] During this inflammatory response, or state of temporary

healing, fibroblasts and lipoblasts are involved in the healing process by synthesizing and secreting various intracellular materials such as the collagen networks, which form the preliminary complexes unto which the healing tissues mature. Neovascularization brings blood vessels to the site and soft granulation tissue is formed. This granulation tissue gets resorbed by macrophages and replaced by a new colony of cells which include chondroblasts and osteoblasts. These cells mineralize the extracellular organic matrix of cartilage and woven bone to create a callus. [74] The osteocyte response and the callus itself prompts the basic multicellular unit (BMU) to increase osteoclastic activity (activation stage) and subsequently osteoblastic activity. The presence of mature osteoclasts leads to a retraction of the bone lining cells from the bone surface, allowing the osteoclasts to bind. Howship's lacunae are formed as a result of the osteoclastic bone resorption. Collagen fragments are removed by specialized cells (reversal stage) and osteoblasts deposit type 1 collagen osteoid matrix, which serves as a scaffold for hydroxyapatite crystal deposition (formation phase). Although, most osteoblasts will die off, however a small proportion of them will remain embedded in the newly formed bone as new osteocytes (or will remain on the surface as bone lining cells). When the formation phase is finished, the bone enters a quiescent phase, where remineralization will continue. [75]

### **1.5 Animal Models in Translational Research**

Studies of bone modeling/remodeling and bone graft healing require experimental subjects with human-like bone and therefore rats (*Rattus norvegicus domestica*) were

chosen as their bones are sufficiently similar to those of human beings. We chose the Sprague-Dawley rat, an outbred strain used extensively in medical research and a tibial model of bone healing was selected based on its size relative to our available surgical armamentarium and materials and its location allowing for easy surgical access.

## **1.6 Neural Networks & Deep Learning**

*Artificial intelligence* (AI) refers to “the ability of a digital computer or computer-controlled robot to perform tasks commonly associated with intelligent beings”. [76] AI has undergone a massive evolution in bridging the gaps between the capabilities of humans and machines. Researchers and doctors alike, work on numerous aspects of the field to make remarkable things happen, such as image and video recognition, image analysis and classification, recommendation systems, natural language processing, etc. And AI research within medicine and dentistry is growing *rapidly*. In 2016, healthcare AI projects attracted more investment than AI projects within any other sector of the global economy, [77] and these numbers are expected to continue to grow especially after the COVID-19 pandemic.

*Machine learning* is a subset of artificial intelligence where a computer can learn and improve its assigned task utilizing internal statistical techniques (vs. human programming). [78] *Deep learning* is a subset of machine learning in which artificial neural networks can learn from data and can extract various features available/present within the data set(s). Artificial neural networks are designed to recognize patterns in

data. They are modelled after the biological neural networks that are found in animal brains. [79] Convolutional neural networks (CNN) are specialized for image recognition tasks. These CNNs can “learn” to perform a variety of tasks by being shown human-engineered examples, instead of being programmed to do such tasks or relying only on statistical methodology. Deep CNNs have demonstrated excellent image classification capabilities, impressive problem-solving abilities, and are believed to hold a large place in future dental/medical research. [80]

#### *1.6.1 From 2D to 3D $\mu$ CT Analysis*

Three-dimensional micro-computed tomography ( $\mu$ CT) is a powerful tool for visualizing and analyzing 3D structures.  $\mu$ CT data typically contains approximately 600 to 1,000 slices of data and this data can be manipulated and used for virtual sectioning and measuring through image stacks. Working with bone/hard tissue samples, sectioning and staining them for histology is inarguably a very time-consuming and technique-sensitive task. With  $\mu$ CT scans, one can easily view hundreds of different slices of a sample virtually – in a matter of hours, in a way visualizing more than what you may see after histological sectioning. Although histological analysis is the gold standard for biological research, it is indeed an arduous process and  $\mu$ CT data is able to predictably yield quantifiable data to study and analyze, especially with calcified hard tissue (bone) samples. For this study, given the project’s goals and time constraints, micro-computed

tomographies were used for data collection and analysis after various hard tissue augmentation techniques were tested in a rat tibia model.

The selection of resolution for  $\mu$ CT imaging is a critical parameter that, if not chosen adequately, would have high impact on the data collection/segmentation results. Since we are studying healing in rat bones, it is important to choose a pixel/voxel size that can adequately capture the rat's trabecular architecture. For most human samples, a resolution of 20-60  $\mu$ m is most often recommended. For mice, 5-10  $\mu$ m is typically used, and for rat samples 10-35  $\mu$ m is typically recommended for easily analyzable cortical and trabecular bony architectures.[81] Hence, for this study, a scanner resolution of 10.043  $\mu$ m (pixel size) was used to ensure an appropriate amount of detail in our scans. Properly segmented  $\mu$ CT data of a bone sample can provide us various 2D and 3D data (based on traditional static bone histomorphometry) such as isolated areas/volumes of interest, different three-dimensional structural parameters, bone thickness, bone porosity, and various bone areas/volumes for both cortical and woven bone.

In order to study the *quantitative* differences in our samples, the total volume of newly formed bone over the recipient site(s) was isolated from our  $\mu$ CT data and a normalized ratio of new bone formation to total bone volume (%) was used for comparison. This selection was based on architectural differences of the various bone grafts tested (*Xenograft, Alloplast, Collagen*) and variations in the available regions of interests (ROI)\* for each group studied (*Bur, Piezo, Onlay*). This metric (%) has been

reported as a valid system of measurement when assessing new bone formation in similar  $\mu$ CT experimental models. [82] [83] [84] To analyze the *qualitative* differences in our samples, the total porosity (%) of the newly formed bone was measured for each sample (per SCP) and compared with the total porosity of the native cortical bone of a *Control* group. One method to collect such data would be to manually segment all slices of data, however this would be too tedious and time consuming given that each scan has approximately 1,000 slices of data. This would require ~40,000 manual segmentations. Since  $\mu$ CT scans of bone samples have varying ranges of radio-opacities, various thresholding techniques (such as Otsu's method) or Hounsfield units could be used for segmentation, however due to the complexity and multi-class nature of our samples, we need more than feature-detection based on radiographic intensity. [85] [86] [87] A blend of human intelligence with the speed of a computer was required for this task and machine learning was the answer.

*\*NOTE: This method will be further explained in CHAPTER 2.*

We designed a powerful convolutional neural network (CNN) algorithm that was created and adapted from other work done with our research group (Di Battista, 2020) to segment the features of interest accurately and effectively from each of our samples to be analyzed. The segmented outputs from our algorithm are an overlay on top of the raw  $\mu$ CT data and do not affect the raw data in any way.

### *1.6.2 The Convolutional Network (CNN)*

The goal for neural networks is to enable machines to view and perceive things as humans do, and use the knowledge for a multitude of tasks such as image recognition, image analysis, and image classification in medical research. The advancements in this scope combined with deep learning has been developed and refined with time, primarily with one particularly successful algorithm – the convolutional neural network (CNN). As mentioned before, the architecture of a CNN is analogous to that of the connectivity pattern of the neurons in the visual cortex. Individual neurons respond to stimuli only in a restricted portion of the visual field (the receptive field). And a compilation of these portions overlap to cover the entire visual area.

A simplified outline of the convolutional neural network structure is illustrated in *Figure 1*. The CNN is a deep learning algorithm which can take an input volume, assign importance (learnable weights and biases) to various aspects/areas in the volume and differentiate one from another. The pre-processing required with a CNN is much lower as compared to other classification algorithms. [88] Initial stages of developing a CNN involve hand-engineered training data, but with enough training CNNs can effectively learn these characteristics and output features of interest for statistical analysis.

A “well-trained” CNN can effectively capture the spatial and temporal dependencies in a volume through the application of relevant filters. The architecture performs a better fit to the volume dataset(s) due to the reduction in the number of

parameters involved and reusability of weights. In other words, the network can be trained to understand the complexity and patterns of the image.

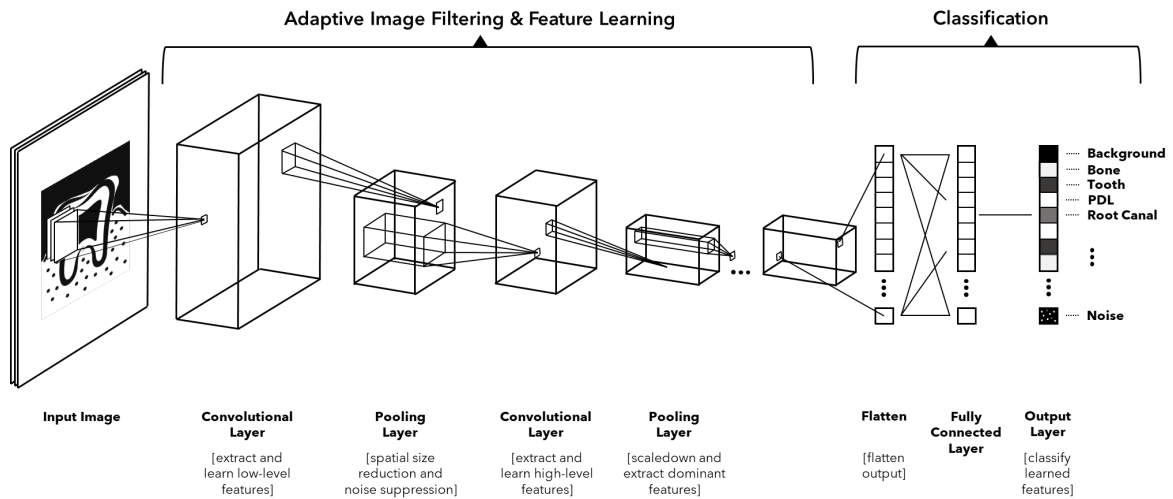


Figure 1. Convolutional neural network structure.

A CNN will extract low level features (major edges, color, gradient orientation, etc.) from an input dataset into a form which is easier to process, without the loss of details critical to getting an accurate segmentation. By adding pooling layers, the spatial size of the convolved feature is reduced, and the computational power required to process the data is decreased significantly through dimensionality reduction. The pooling layers will also extract dominant features from your samples (rotational and positional variations) and further convolutional layers will then extract the higher-level features (minor edges, etc.) as further pooling layers will reduce noise. [89] This training continues until the network has a completed understanding of the volumes/areas in the

dataset(s). For more complex volumes, the number of convolutional layers and pooling layers needs to be increased as does the computational power. [90]

After completing a series of epochs in the above process, the final output is flattened and fed into a regular neural network for classification and multi-level feature extraction purposes. Our multi-class segmentation (Original Cortical Bone, New Bone Formation, Background, Bone Graft Material) algorithm utilized a U-net training architecture [85] and each sample had value accuracy rates of over 99.9% when self-verifying after training. After whole scans were segmented, the results were optimized by hand with another trained researcher to ensure that no features were mis-labelled.

## RESEARCH PROJECT

### *Hypothesis*

We hypothesized that selective cortical penetrations created using the piezoelectric knife may lead to enhanced cellular activation during bone regeneration when compared with conventional bur or subperiosteal onlay grafting methods.

### *Aim*

The aim of this project was to evaluate and compare both the quantity and quality of new bone formation when using a piezoelectric knife (versus the conventional bur and onlay grafting techniques) to prepare the recipient site in bone regeneration surgery. This was done by using X-ray microscopy and deep learning analysis.

## CHAPTER 2

### MATERIALS AND METHODS

#### 2.1 Outline

The study was approved by the Boston University Medical Center Institutional Animal Care and Use Committee (BUMC IACUC) for all animal procedures in this study (protocol AN-15682). The animals were purchased from Charles River Laboratories International and acclimatized in the Laboratory Animal Science Facility for at least 2 days (48 hours) prior to performing the surgeries. A total of 20 Sprague-Dawley male rats were used. The animals were all 9-to-10-week-old male Sprague-Dawley rats, weighing around 300-350g. The pre- and post-operative weights of the rats, their levels of pain/distress, and hematological data at the time of sacrifice were closely monitored at Boston University's Animal Science Center in order to assess the peri-experimental systemic health of the rats. The experimental groups were divided as follows: A piezoelectric knife (BS1 insert) of the Piezotome 2 (Satelec®, Acteon group, Merignac, France) was used to create four selective cortical penetrations (trans-cortical) on the tibia and the recipient sites were grafted using either a *Xenograft* (Bio-Oss Collagen, Geistlich Biomaterials), an *Alloplast* (OsteoGen, Implants Ltd), or a *Collagen* matrix (Mucograft, Geistlich Biomaterials) (*PIEZO group*). A conventional surgical carbide bur (#1/2) was used to create four selective cortical penetrations (trans-cortical) on the tibia and the recipient sites were grafted using either a *Xenograft*, an *Alloplast*, or a *Collagen* matrix (*BUR group*). The defects created using a surgical carbide bur were done using a template to ensure that they were similar in size and shape to those created by the BS1

Piezotome insert (Figure 14). A full-thickness envelope flap was created on the tibia with no bone intervention and the recipient sites were grafted using either a *Xenograft*, an *Alloplast*, or a *Collagen* matrix (*ONLAY* group). For the *Xenograft* study groups, special attention was directed to the SCPs on the right tibias and the onlay grafts on the left tibias. For the *Alloplast* and *Collagen* study groups, the surgical techniques were mixed on left/right sides. The rats in the experimental groups were euthanized with 20% CO<sub>2</sub> asphyxia at 28 days post-operatively. Tissue and serum responses were assessed at 28 days. Three 12-week-old male Sprague-Dawley rats with untouched tibias were euthanized and served as controls (*Control* group).

## **2.2 Grafting Material Preparations**

The three grafting materials were prepared under sterile conditions.

### *2.2.1 Xenograft*

For the *Xenograft* groups (Bio-Oss Collagen, Geistlich Biomaterials), a 100 mg preformed block was used. (Figure 2) The block was held in place using sterile cotton pliers and a sterile stainless-steel blade (#15, Bard Parker) was used to create the individual xenograft blocks. (Figure 3) A sterile periodontal probe was used as a guide to create individual xenograft blocks 6 mm in length, 3 mm in height, and 3 mm in width. (Figure 4). The grafts were hydrated with sterile saline prior to surgical placement, per the manufacturer's recommendations.



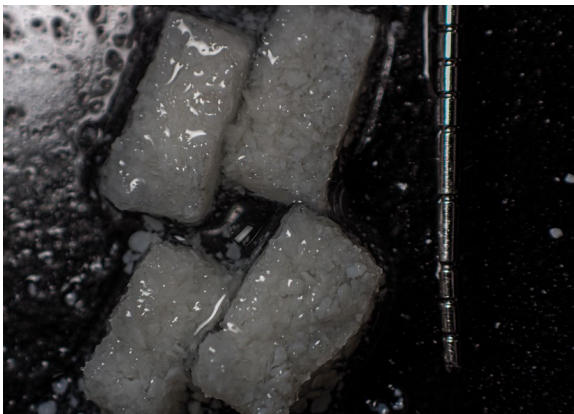
**Figure 2. Xenograft group.**

This figure shows the dehydrated 100 mg preformed xenograft block, prior to cutting.



**Figure 3. Xenograft preparation.**

This figure illustrates how the xenograft block grafts were prepared using sterile cotton pliers, surgical 15 blade, and periodontal probe.



**Figure 4. Xenograft dimensions.**

This figure shows the final dimensions of the xenograft block grafts hydrated in sterile saline. (~ 6 x 3 x 3, mm)

### 2.2.2 Alloplast

For the *Alloplast* group (OsteoGen, Implants Ltd), a 20 mm x 40 mm x 3 mm strip was used. (Figure 5) A 6mm sterile, disposable biopsy punch (Integra Miltex, Fisher Scientific) was used to create equally sized alloplast discs. (Figure 5) The discs were 6 mm in diameter and 3 mm in thickness. (Figure 6) The grafts were not hydrated with sterile saline prior to surgical placement, according to the manufacturer's recommendations.

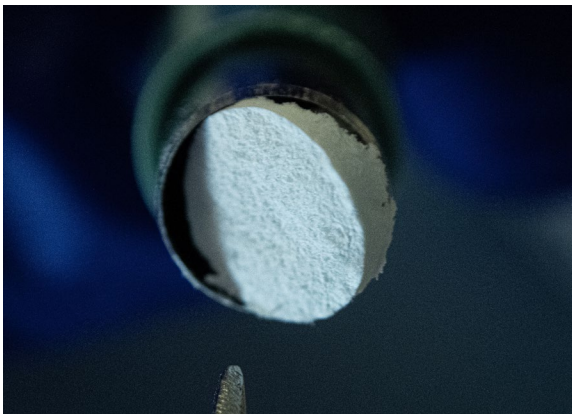
### 2.2.3 Collagen Matrix

For the *Collagen* matrix group (Mucograft, Geistlich Biomaterials), a 30 mm x 40 mm x 3 mm strip was used. (Figure 7) A 6 mm sterile, disposable biopsy punch (Integra Miltex, Fisher Scientific) was used to create equally sized collagen matrix discs. (Figure 8) These discs were 6 mm in diameter and 3 mm in thickness (Figure 9), as well. The grafts were not hydrated with sterile saline prior to surgical placement, according to the manufacturer's recommendations.



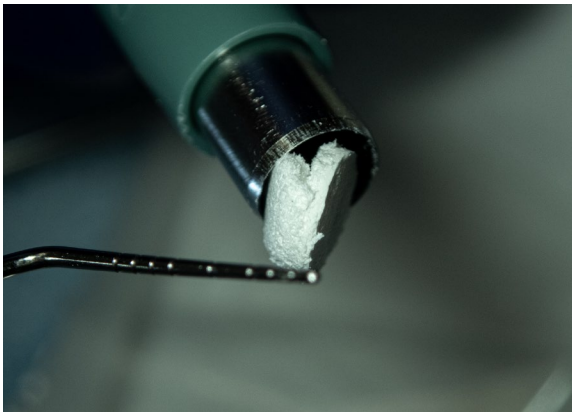
**Figure 5. Alloplast group.**

This figure shows the alloplast bone strip after the individual alloplast discs were harvested.



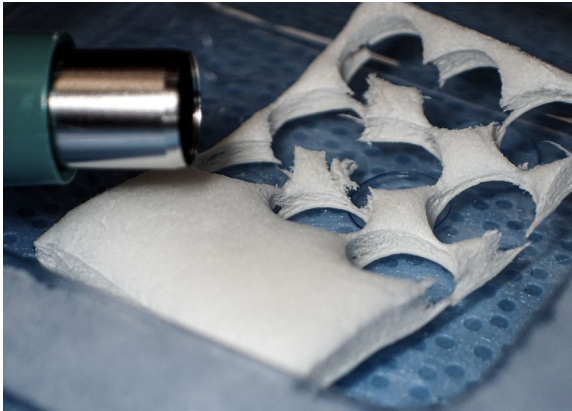
**Figure 6. Alloplast preparation.**

This figure illustrates how the alloplast discs were prepared using a 6 mm sterile biopsy punch.



**Figure 7. Alloplast dimensions.**

This figure shows the final dimensions of the alloplast discs prior to surgical placement. (6 x 3, mm)



**Figure 8. Collagen matrix group.**

This figure shows the collagen matrix after the individual alloplast discs were harvested.



**Figure 9. Collagen matrix preparation.**

This figure illustrates how the collagen matrix discs were prepared using a 6 mm sterile biopsy punch.



**Figure 10. Collagen matrix dimensions.**

This figure shows the final dimensions of the collagen matrix pucks prior to surgical placement. (6 x 3, mm)

### 2.3 Surgical Procedure

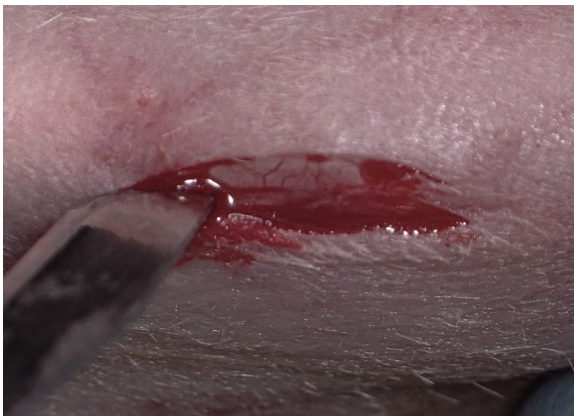
The animals were fed rat chow and water ad libitum and weighed daily. They were purchased from the vendor and given at least 48 hours to acclimate in the housing area of the Boston University Animal Science Center (ASC) prior to experimentation. They were housed two to a cage. At the time of surgery, the rats were sedated and anesthetized with intraperitoneally-administered ketamine (75-95 mg/kg) and Xylazine (5mg/kg) in one bolus injection. Tail-pinch test was performed to confirm depth of anesthesia prior to procedure. 0.3 mg/kg of subcutaneously-administered sustained-release buprenorphine was given post-operatively for extended-release pain control.

Surgical site preparation began with shaving the mesial surfaces of each rat's legs. An electric clipper was used until adequate skin exposure was achieved. The skin was sanitized with an isopropyl alcohol solution (70%). The leg was then extended and held in place while an incision was made using a sterile, stainless steel blade (#15, Bard-Parker). (Figure 11) The recipient site was accessed through this full-thickness incision (Figure 12) and was created by raising a full-thickness, "pouch"-like envelope flap. While reflecting the periosteum, special care was taken to avoid damage to the *Tibialis Anterior* muscle. If no selective cortical penetrations are planned, the grafting material would be placed under the flap at this time (*ONLAY group*). (Figure 13)



**Figure 11. Surgical site preparation.**

Full thickness incision is carefully carried out in contact with the shaft of the tibia.



**Figure 12. Incision design.**

Incision is made with a 15C sterile stainless-steel blade.



**Figure 13. Full-thickness envelope flap.**

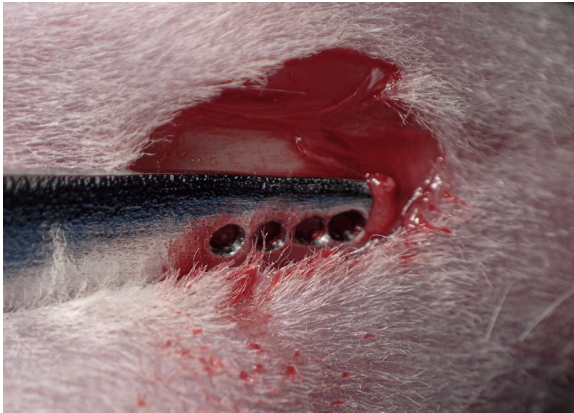
Full thickness mucoperiosteal envelope flap is raised using microsurgical instruments. At this point, if no selective cortical penetrations are planned, the grafting material is placed. (*ONLAY* group)

If selective cortical penetrations are planned with a surgical carbide bur (#1/2) (*BUR group*), a custom-made surgical stent was used. This stent was designed with channels of similar size and shape to those created by the BS1 Piezotome insert. (Figure 14) The template is held firmly in place along the shaft of the tibia. (Figure 15) Four [trans-cortical] selective cortical penetrations were created through the channels of the surgical template using an Osseocision dental motor system (Biomet 3i) with a surgical stainless steel carbide bur (#1/2). (Figure 16) The handpiece motor was run at 800 RPM speed with a torque of 20:1 under copious irrigation with sterile saline. Penetration of through the cortical layer of bone was confirmed by visual observation of bleeding from the surgically-created bony defects.



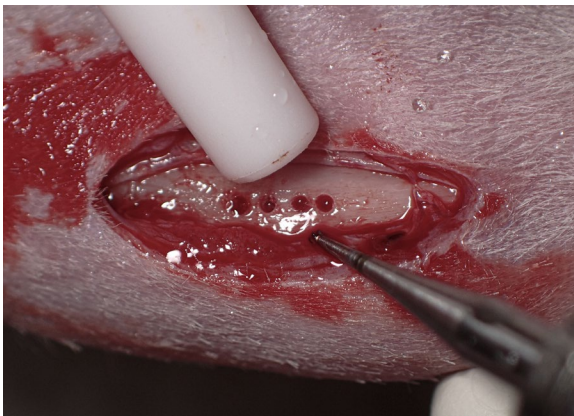
**Figure 14. Armamentarium.**

Surgical carbide bur (#1/2) (*BUR group*), custom-made surgical template, piezoelectric knife (BS1 insert) (*PIEZO group*). If selective cortical penetrations are planned, the carbide bur is used with a surgical template designed to create equally-spaced SCP's.



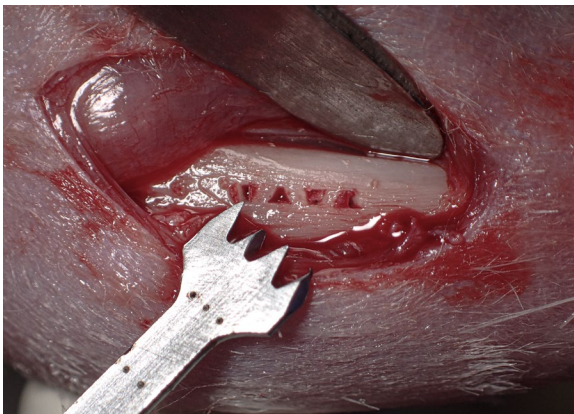
**Figure 15. Surgical template placement.**

Custom-made surgical template is firmly held in place along the bone surface inside the full-thickness envelope flap.



**Figure 16. Four selective cortical penetrations created with a surgical carbide bur.**

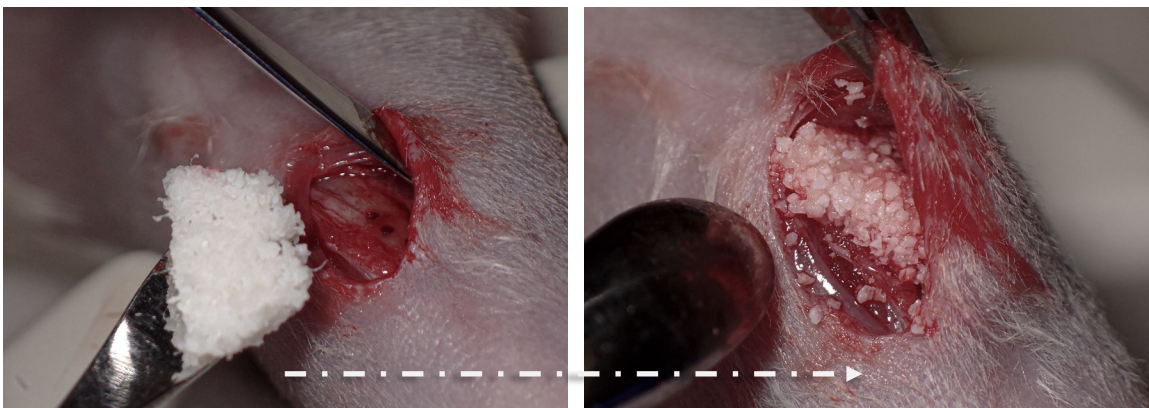
Four SCP's are created with a bur (#1/2) using the surgical template with sterile saline irrigation. The carbide is run at a speed of 800 RPM. Note the bleeding from the marrow space confirming trans-cortical penetration.



**Figure 17. Four selective cortical penetrations created with a piezoelectric knife.**

Four trans-cortical SCP's are created using the piezoelectric knife (BS-1 insert).

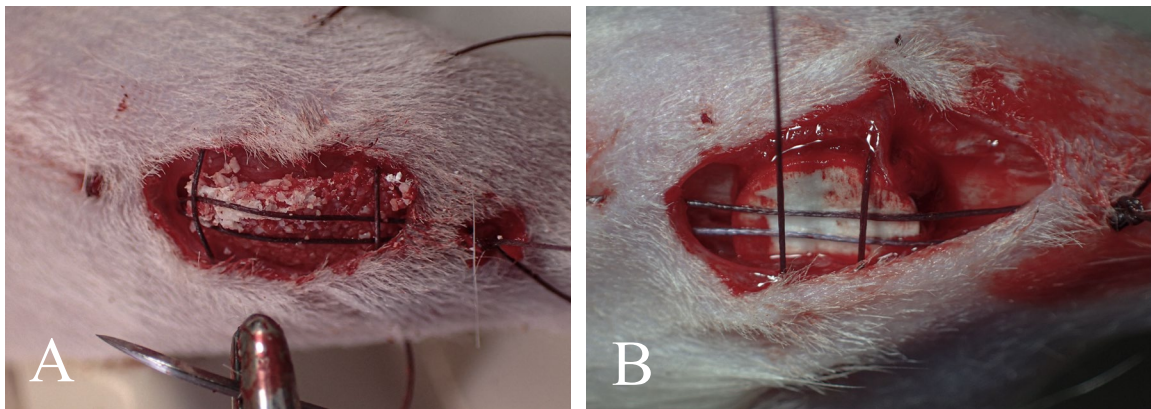
If selective cortical penetrations are planned with a piezoelectric knife (BS1 insert) (*PIEZO group*), four selective cortical penetrations are made using a Piezotome II unit (Acteon, Satalec). The settings used were modulation of 30 Hz and an irrigation setting of 60 mL/min sterile saline. The four teeth of the piezoelectric knife are inserted until bleeding is confirmed (approximately 1 mm transcortical). Special attention was made to not insert the BS1 insert past the base of the four cutting edges, in order to create four separate selective cortical penetrations. (Figure 17) The grafting materials were then placed under the full-thickness envelope flap using microsurgical instruments and magnification. In recipient sites where selective cortical penetrations were made, the grafting material was placed directly centered over the four cortical defects. (Figure 18) Using three different grafting materials (xenograft, alloplast, collagen matrix) and three different recipient bed preparation techniques (bur, piezo, onlay), this yields 9 different bone grafting techniques (nine experimental groups).



**Figure 18.** Placement of the grafting material into the recipient site.

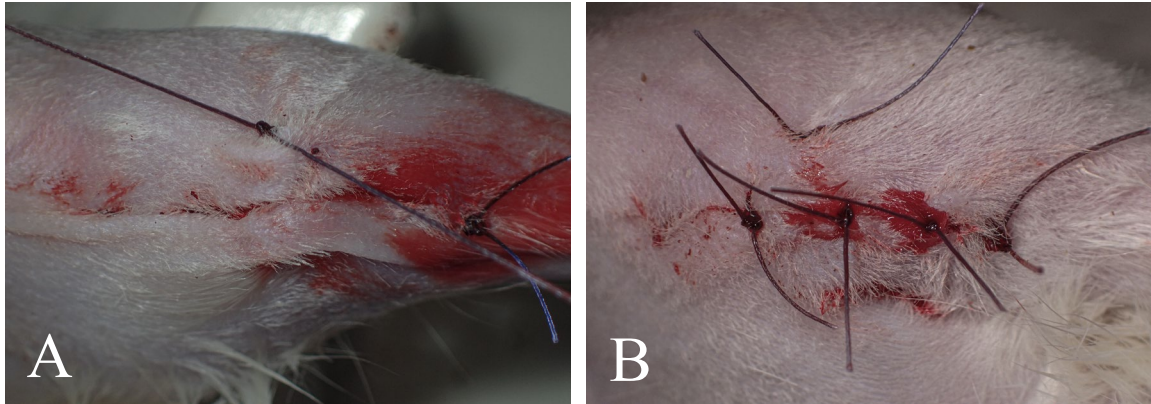
This figure illustrates the placement of the xenograft into the recipient site prepared with a surgical carbide bur (*Bur, Xenograft*).

Each of the three grafting materials were fixed over the recipient bed preparation using two mattress sutures: one vertical mattress suture to prevent axial displacement of the graft and one modified horizontal mattress suture to prevent rotational displacement of the graft. (Figure 19) Once the second mattress suture (horizontal) is tightened, the flap edge, including the periosteum, was brought *over* the grafting material. (Figure 20) Finally, a layer of three single interrupted sutures were added to obtain primary closure of the flap edges. The synthetic, resorbable, braided suture strands used were 4-0 polyglycolic acid (PGA) (ACE Surgical) for the two mattress sutures and 5-0 polyglycolic acid (PGA) (ACE Surgical) for the single interrupted sutures.



**Figure 19. Graft fixation with vertical mattress suturing technique.**

This figure illustrates the fixation of the xenograft (A) and the collagen matrix (B) using two mattress suturing techniques. Both the vertical mattress and the horizontal mattress sutures prevent displacement of the graft from the recipient bed.



**Figure 20. Flap advancement and immediate primary closure.**

(A) The horizontal mattress suture brings the full-thickness flap edge over the grafting material and in close approximation with the other border. (B) Three simple interrupted sutures are tied for immediate, low-tension primary closure.

Animals were allowed to fully recover from anesthesia in their cage over a warm water-filled heating pad with close monitoring. 3 mg/kg of subcutaneously-administered sustained-release Buprenorphine (SRLAB, ZooPharm) was given for 72-hour extended pain control. Once the rats were fully awake, they were placed in their housing room. Their weights and condition were monitored daily for the first 14 days and once weekly until day 28.

At day 28, after euthanization by 20% carbon dioxide asphyxiation, blood was collected from the heart for hematological analysis. The skin was carefully dissected around both legs and the legs (femur, knee, and tibia) were removed at the acetabulofemoral joint. Soft tissues around the tibia were kept intact. The limbs were preserved in 4% paraformaldehyde (PFA) solutions and stored at 4°C until scanning.

## 2.4 3D X-ray Microscopy

A high-resolution X-ray microtomography imaging system (Xradia 520 Versa, Zeiss) was used to scan each sample at Boston University's Photonics Center (PHO). This 3D microscope can capture highly accurate non-destructive 3D images utilizing advanced X-ray source technology and high-resolution detectors. This X-ray computed tomography technology has been used in life science research and in advanced material research and development. It is capable of capturing a wide array of contrasts, which allows imaging of hard tissues and less-calcified tissues as well. For this study, a scanning resolution of 10  $\mu\text{m}$  voxel size was used, as previously mentioned.

The Scout-and-Scan Control System workflow (Zeiss) was used to localize the volume of interest along the tibial diaphysis and acquire our tomographies. (Table 1) First, the sample's data folder was set and a new recipe creation was initiated. Second, using the visual light camera, the sample was loaded, roughly positioned on the axis of rotation, and the X-ray source and detector was positioned. (Figure 21) Third, the sample's region of interest (ROI) and field of view (FOV) was located, and the imaging parameter values were set. The region of interest (tibia diaphysis) was found and centered at low magnification. The ROI was then fine-tuned at high magnification. The X-ray source and detector positions were set. The appropriate source filters and voltages were set. The acquisition time was then determined. Fourth, the recipe's 3D scan parameters were set up. Lastly, the recipe was run and the tomographies were acquired.

| MICROTOMOGRAPHY IMAGING SETTINGS |                      |
|----------------------------------|----------------------|
| Source Voltage                   | 80.00 <i>kV</i>      |
| Source Power                     | 7.00 <i>W</i>        |
| Approximate Source Position      | -50.00 <i>mm</i>     |
| Detector Position                | 205.00 <i>mm</i>     |
| Scan Duration                    | ~2 <i>hours</i>      |
| Objective Magnification          | 0.4 X                |
| Field Mode                       | Normal               |
| Source Filter                    | LE1                  |
| Pixel Size                       | 10.043 $\mu\text{m}$ |

Table 1. Scout-and-Scan Control System settings for  $\mu$ -CT.

This table outlines the scan settings for the Scout-and-Scan Control System (Xradia 520 Versa, Zeiss).



Figure 21. Volume of interest localization using 3D x-ray microscopy.

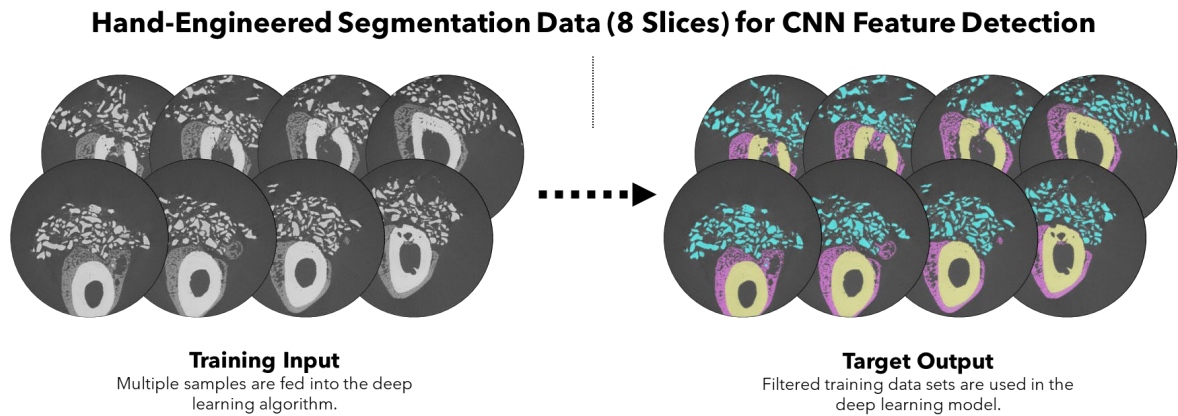
This figure illustrates the technique used for localizing and scanning the samples. Note the image on the right, which shows how the samples were loaded into the scanner using a custom-made falcon tube base.

## **2.5 Deep Learning**

### *2.5.1 Training Input*

As discussed earlier in the introduction, for our project, we did not have the time nor manpower to segment each slice of every micro-CT scan individually, so we elected to design and use a deep learning algorithm for segmentation and data extraction. Various software packages can be used for this type of CNN design, however we used Dragonfly (ORS Systems) because of its wide range of image analysis capabilities and ease of use. First, we hand segment 8 slices of every scan to feed our algorithm. (Figure 22) These “ground truths” were verified with another experienced investigator. This is the “example” that we give to the computer that we will feed the training model in order to allow for it to “learn” as mentioned previously.

The purpose of this step is to provide our model with a learning set containing the original, unaltered micro-CT scans (“what we are beginning with”) and also to provide our segmented target output to show the algorithm “what we want to end up with”.



**Figure 22. Deep learning input data.**

Figure 22 shows a simplified version of the input that is fed into the preliminary convolutional neural network algorithm. Several iterations of the training data are layered for feature detection and deep learning modeling.

### 2.5.2 Other Modelling Parameters

Training a deep learning algorithm can take weeks if not months depending on the optimization algorithm used and the computational power available. For our project, we elected to use a CNN as discussed earlier and the method for optimization utilized was the AdaDelta algorithm. This unique learning rate method helps minimize the value of the function loss by dynamically adapting its learning memory overtime with scaled down datasets (a decaying average of all past squared gradients) and therefore has remarkably minimal computational overhead. [91]

Minimizing function loss is analogous to minimizing error in our deep learning model - the more error in our algorithm, the less accurate our segmentation outputs will be. And loss functions are used to calculate how well our deep learning model is

modelling given the 8 slices of training data. For our project we have a multi-level segmentation target output (new bone, original bone, bone graft, and background), therefore our algorithm's errors can be multi-level. For feature recognition, the problems we face are mostly binary on each individual feature level. For example, if our deep learning algorithm tags a particular pixel as new bone, when in reality it is a bone graft particle, this needs to be recognized as an error based on our training data sets. [92]

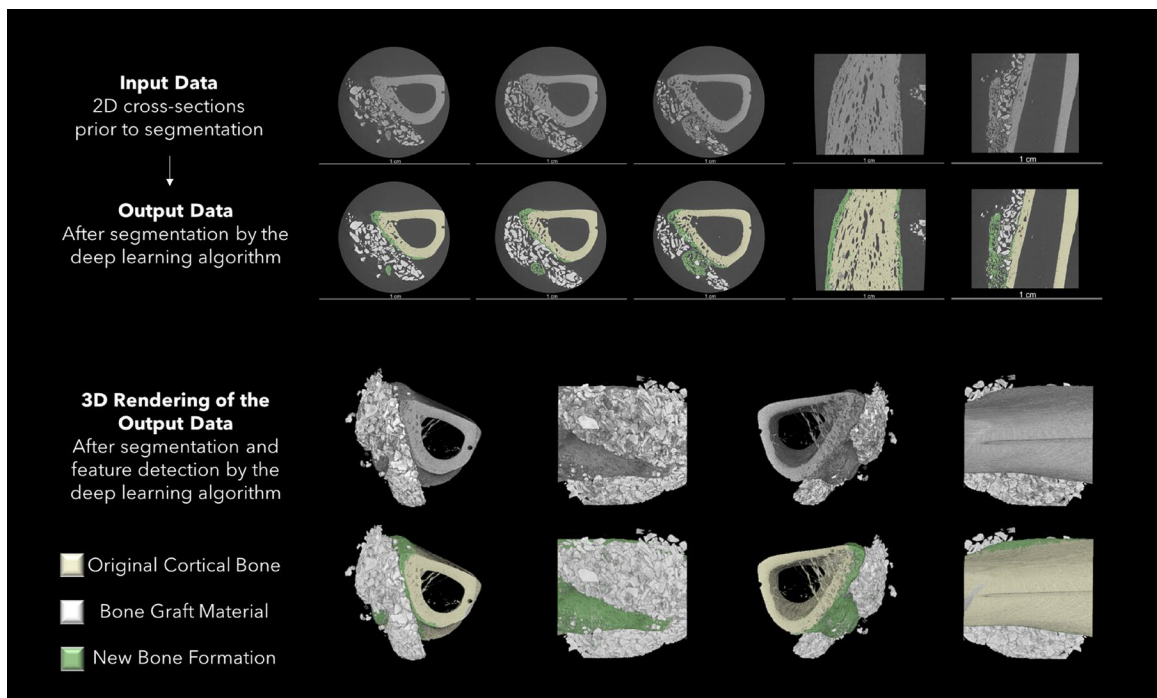
Categorical cross-entropy is the loss function model chosen for our multi-label segmentation because researchers on our team have had accurate segmentation results with this method.

| <b>DEEP LEARNING ALGORITHM MODELING PARAMETERS</b>           |  |
|--|--|
| <b>Architecture</b>  | U-Net (5-level)                        |
| <b>Model Type</b>  | Multi-label Segmentation               |
| <b>Class Count</b>   | 4                                      |
| <b>Input (Patch) Size</b>                                    | 128                                    |
| <b>Stride to Input Ratio</b>                                 | 0.5                                    |
| <b>Epochs Size</b>   | 50                                     |
| <b>Loss Function</b>   | categorical_crossentropy               |
| <b>Optimization Algorithm</b>                                | Adadelta                               |
| <b>Memory Ratio</b>  | < 1.0                                  |
| <b>Data Augmentation Used on Input Data</b>                  | Rotation, Mirroring, Shearing, Scaling |
| <b>Percentage of Training Data to be Used for Validation</b> | 25%                                    |

**Table 2. CNN algorithm modeling parameters used in Dragonfly's deep learning tool.**

This table outlines the modeling parameters for the Deep Learning Tool (Dragonfly, ORS) for reference.

*Table 2* outlines the parameters set for the Deep Learning Tool in the 3D visualization and analysis software application. Note that 50 epochs were used in this algorithm simply because our hardware did not have enough processing power for more. All models, however, had value accuracy rates of over 99.0% when self-verifying after training and a maximum value loss of 0.03% after Epoch 50 out of 50. A sample of the resulting output segmentation from our deep learning algorithm is shown in Figure 23. You can appreciate the successful feature detection of the output data with impressive distinctive abilities between original cortical bone, bone graft material, new bone formation, and background.

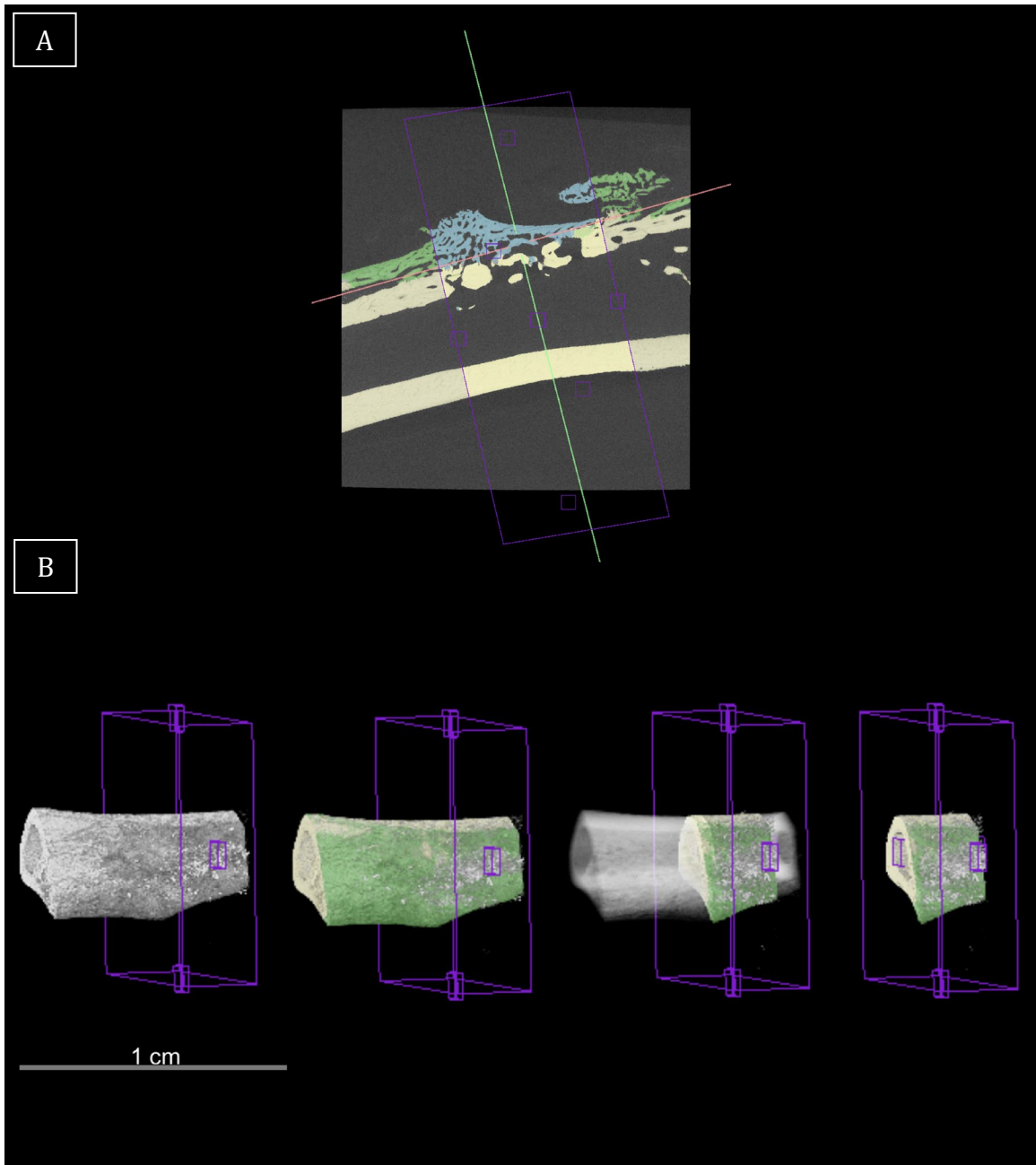


**Figure 23. Example output of the deep learning segmentation.**

The top two rows show a cross-sectional representation of an original sample (onlay technique with xenograft) that is fed through our deep learning segmentation algorithm. The bottom rows show a three-dimensional rendering of the input-output data.

### 2.5.3 Data Acquisition

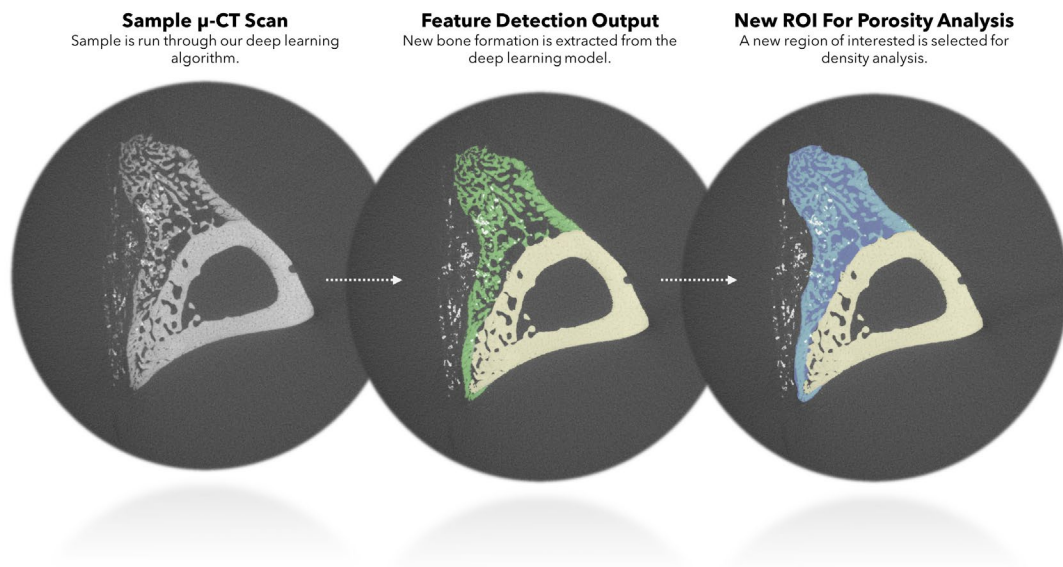
After whole scans were segmented using these algorithms, the resultant segmentations were manually optimized to ensure that no features were obviously mislabeled. This was cross-checked with another blinded, experienced researcher. The output was essentially an overlay on top of the raw data, with no effects on the original data in any way. The models showed successful feature recognition of the original cortical bone with new bone formation around the surgical sites, often with residual bone graft particles encapsulated within the newly formed calcified tissues. The data extracted from the whole scan segmentations, however, included the volume of new reactive bone formation [ $\mu\text{m}^3$ ], the volume of original tibia cortical bone [ $\mu\text{m}^3$ ], and the volume of the graft [ $\mu\text{m}^3$ ]. Since the whole scans were taken at 10  $\mu\text{m}$  resolution and the surgical sites ranged from 3.5 – 6 mm in distance, a region of interest (ROI) needed to be established for each sample in order to extract data from the area where grafting was completed only. Because of the differences in surgical site dimensions and also because 2 of our 20 scans were out of alignment (missed 2 of the 4 SCPs), the ROI could not be standardized across all groups. The ROI was defined based on the following criteria: (1) When SCPs were used, the ROI must be within the beginning of the first defect and the end of the last defect, (2) If identifiable, the graft must be within both ends of the intracortical defects within the range described above (underlying the graft), and (3) When no decortications were used (*Onlay* group), the ROI was defined as the tibial volume above which grafting material was overlaid. Figure 24 shows an illustration of how the ROI was selected for each sample.



**Figure 24. Region of Interest (ROI) Isolation**

Figure 24 shows a cross sectional view of a region of interest (in purple) highlighted around the four selective cortical penetrations. Figure B illustrates a three-dimensional view of the ROI defining steps. Note each ROI is selected perpendicular to the long axis of the tibial diaphysis.

Once again, when conducting our quantitative analysis of the newly formed bone for each study group, due to the differences in size and architecture of our volumes of interest (ROIs), the data was normalized for each sample by calculating a ratio of new bone volume to total bone volume. Also, the total porosity of the newly formed bone for each study group plus the 3 control samples was calculated using a technique illustrated in Figure 25. For porosity analysis, a new ROI needed to be created (the area or volume of space occupied by the newly formed bone – including the background/marrow spaces – in blue). The deep learning algorithm was not successful when detecting the new ROI for porosity analysis in whole scan samples. So, after the original multi-level segmentation algorithm was run, one slice per SCP was randomly selected to manually create this new ROI. Then the surface area of newly formed bone was divided by the surface area of the ROI to arrive at a density measurement which was easily converted to total porosity in Excel (Microsoft 2013).



**Figure 25. Total Porosity Analysis Example.**

## **2.6 Hematological Analysis**

Blood was isolated from each rat immediately following carbon dioxide asphyxiation by cardiac puncture. [93] The blood was transferred to heparin-coated blood collection tubes (VACUETTE, Thomas Scientific). After centrifugation and isolation, the serum was tagged and frozen at -80°C. Each rat's blood was analyzed using a Hemavet 950 FS blood analyzer (Drew Scientific), using the Mascot hematology profile. All samples were analyzed within 24 hours of collection.

## **2.7 Statistical Analysis**

Statistical analysis was completed using the software JMP Pro 15 (SAS Institute Inc, Buckinghamshire). Analysis of variance (ANOVA) was used. For new bone formation measurements and total porosity, the ANOVA test was utilized with a power of 95%. Statistical differences were considered significant at the 5% critical level. If a statistically significant difference was observed between means, Tukey honest significance test (HST) was applied to each pair to investigate which ones differed significantly. Lastly, the Dunnett's test was used to compare total porosities to the control.

The tables with values for each sample's raw  $\mu$ CT data, raw hematological data, and raw weight monitoring data are available in the *Appendix*.

## CHAPTER 3

### RESULTS

#### 3.1 3D X-ray Microscopy

##### 3.1.1 Segmentation Overview

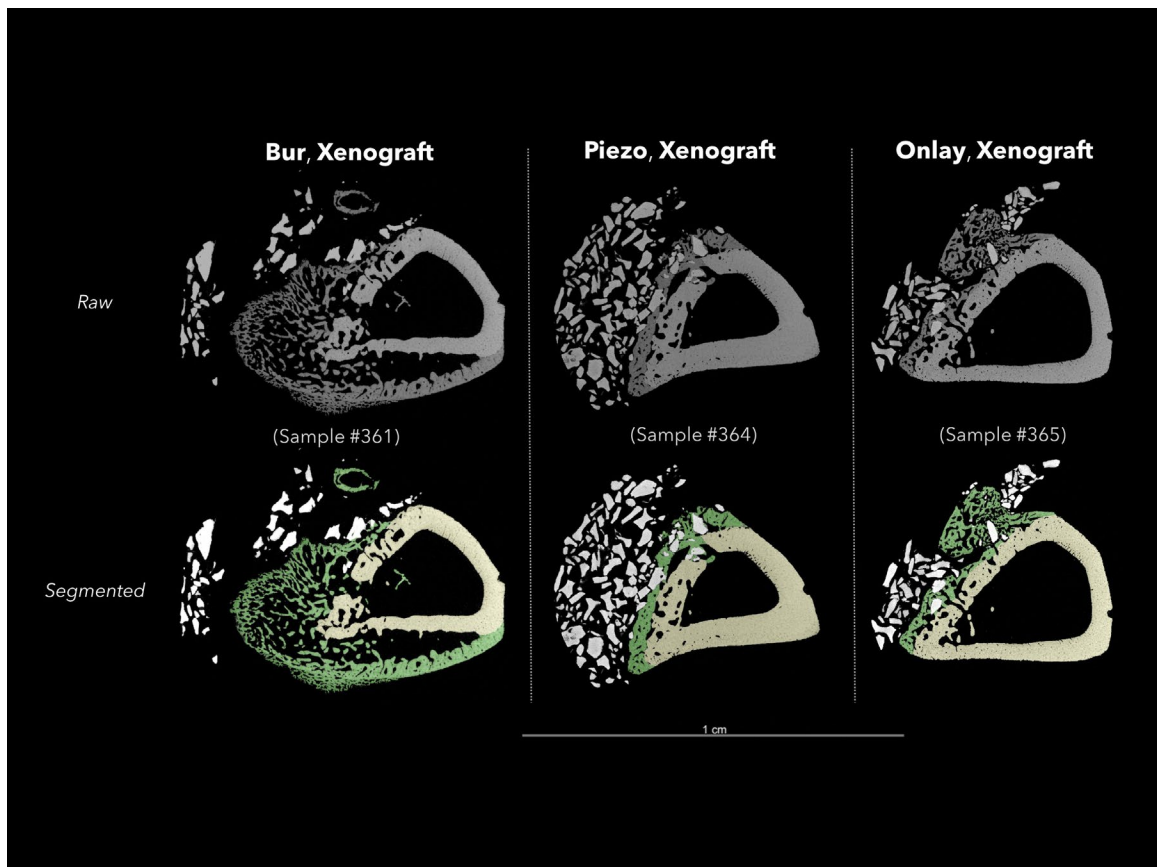
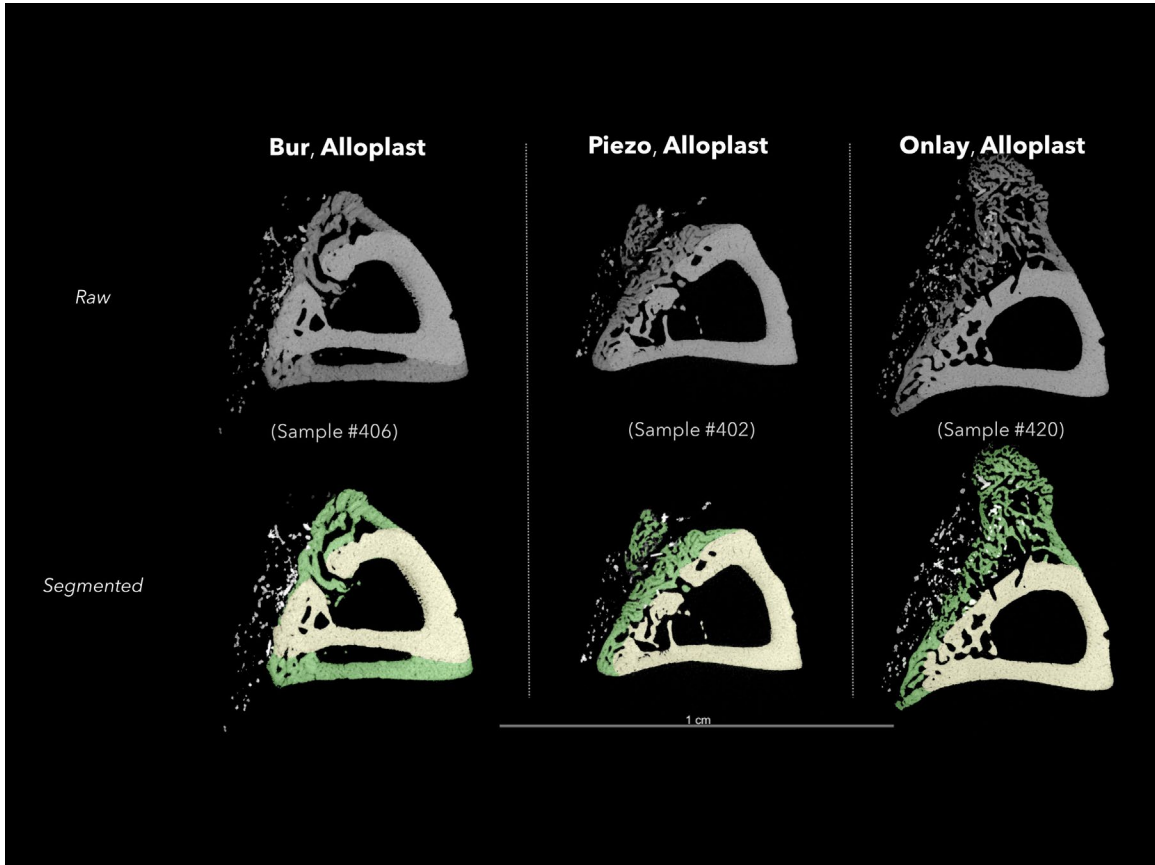


Figure 26. Segmented Xenograft samples.

Figure 26 shows the result of the segmentation for one sample of each *Xenograft* group (*Bur*, *Piezo*, *Onlay*) on a longitudinal cross-section (slice). The original cortical bone is segmented in yellow, the newly formed calcified tissue in green, and the residual bone graft particles are white.



**Figure 27. Segmented Alloplast samples.**

Figure 27 shows the result of the segmentation for one sample of each *Alloplast* group (*Bur*, *Piezo*, *Onlay*) on a longitudinal cross-section (slice). The original cortical bone is segmented in yellow, the newly formed calcified tissue in green, and the residual bone graft particles are white.

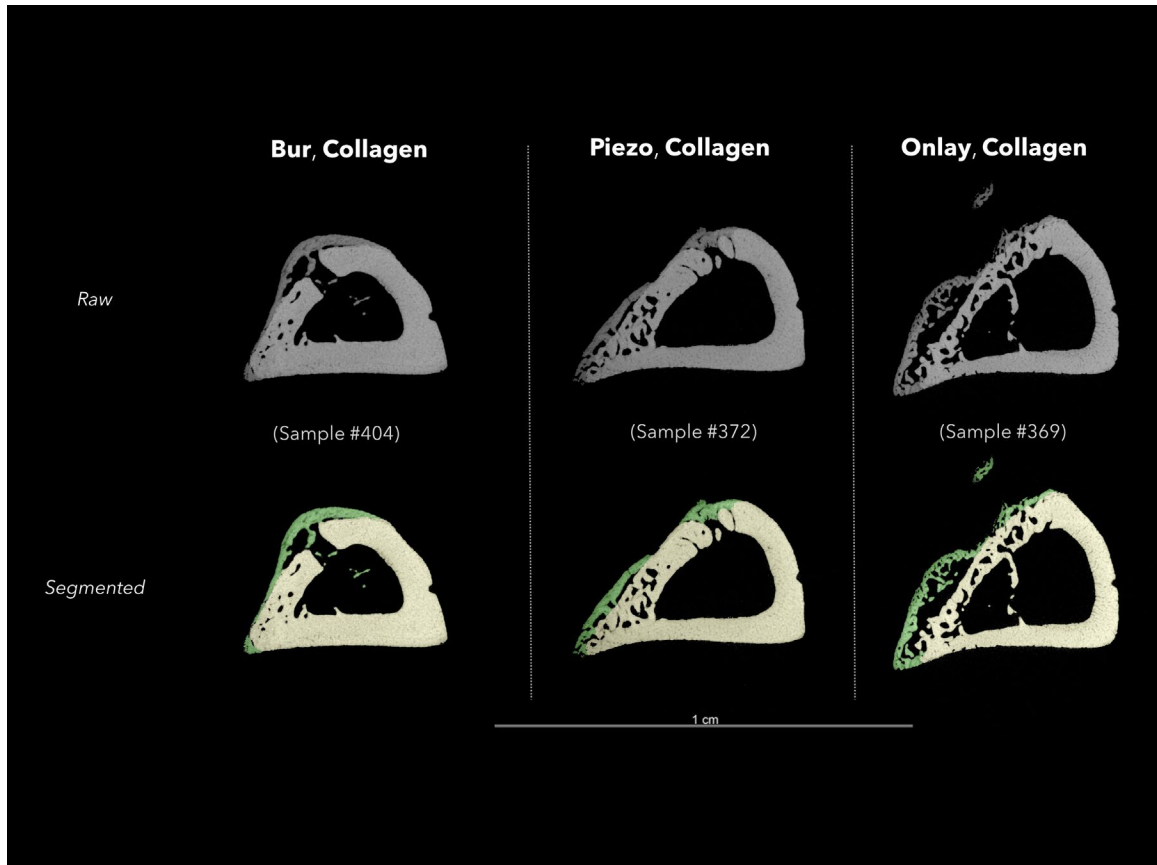


Figure 28. Segmented Collagen Matrix samples.

Figure 28 shows the result of the segmentation for one sample of each *Collagen* matrix group (*Bur*, *Piezo*, *Onlay*) on a longitudinal cross-section (slice). The original cortical bone is segmented in yellow, and the newly formed calcified tissue is green.

### 3.1.2 Volumetric Analysis

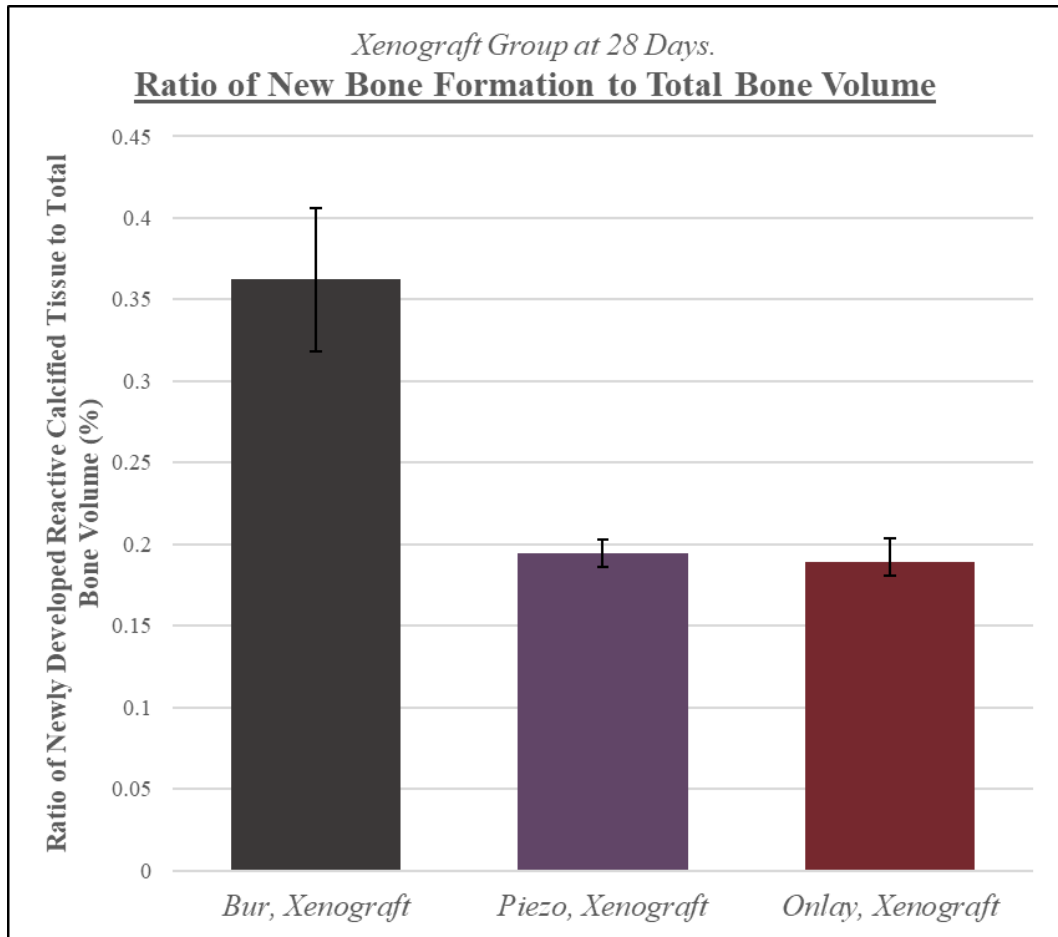
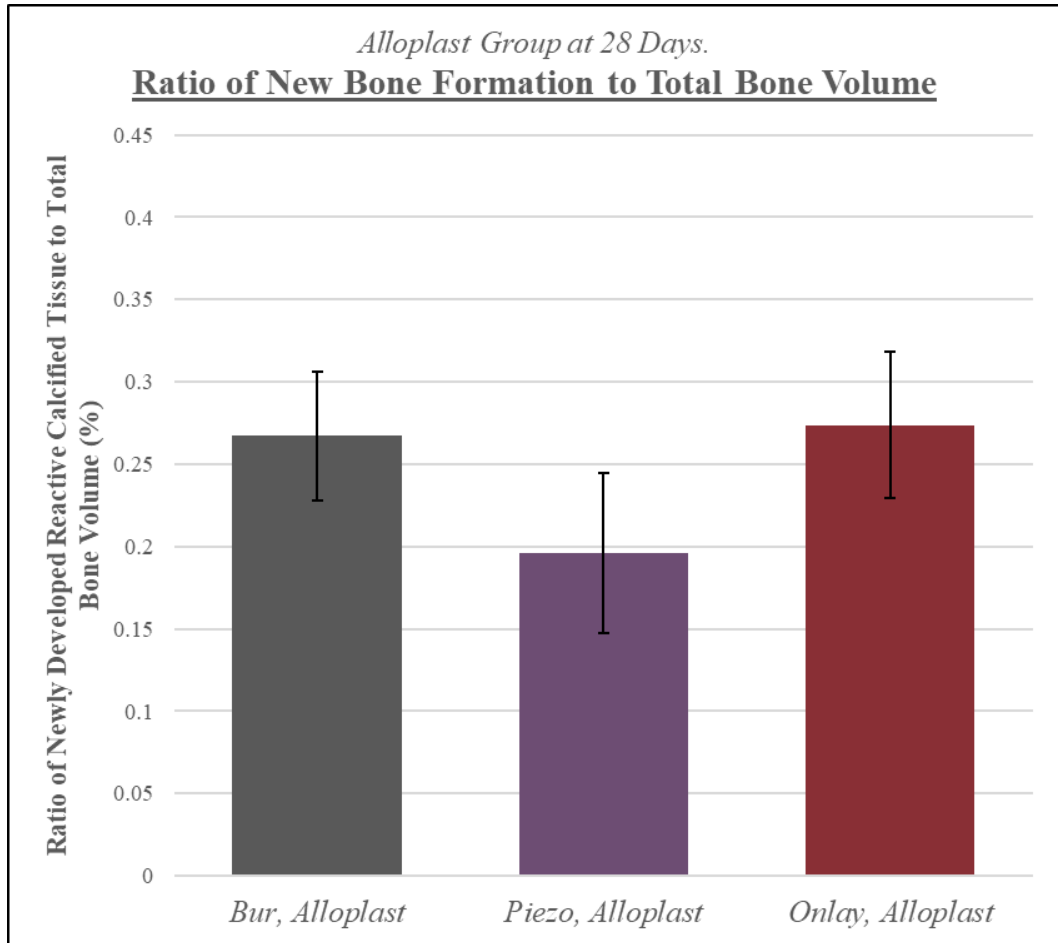


Figure 29. New bone formation data for the xenograft group at day 28.

This figure illustrates the new bone formation data for the 28-day *Xenograft* samples.

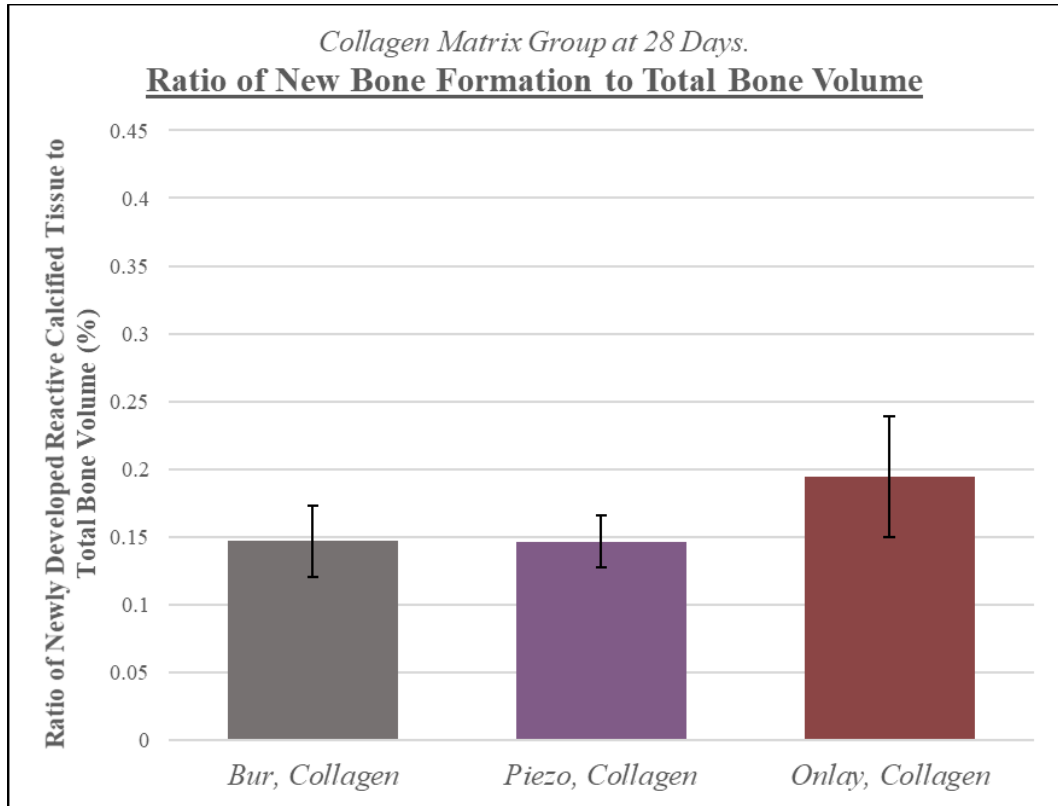
For our “*quantitative*” analysis of the new bone formation in the 28-day *Xenograft* groups, selective cortical penetrations with a *Bur* yielded the highest percentage of new bone formation to total bone volume. Analysis of variance (ANOVA) was used with a power of 95% and a 5% critical level and showed no statistically significant differences between the three groups (*Bur*, *Piezo*, *Onlay*).



**Figure 30.** New bone formation data for the alloplast group at day 28.

This figure illustrates the new bone formation data for the 28-day *Alloplast* samples.

For our quantitative analysis of the new bone formation in the 28-day *Alloplast* groups, selective cortical penetrations with an *Onlay* technique yielded the highest percentage of new bone formation to total bone volume. ANOVA was used with a power of 95% and a 5% critical level and showed no statistically significant difference between the three groups (*Bur, Piezo, Onlay*).



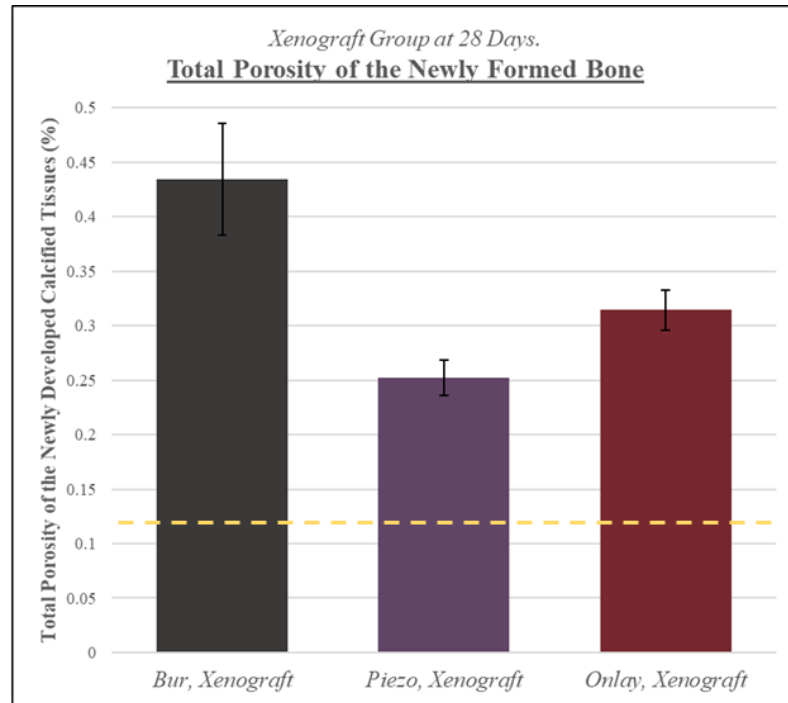
**Figure 31. New bone formation data for the collagen matrix group at day 28.**

This figure illustrates the new bone formation data for the 28-day *Collagen* samples.

For our quantitative analysis of the new bone formation in the 28-day *Collagen* groups, selective cortical penetrations with an *Onlay* technique yielded the highest percentage of new bone formation to total bone volume. ANOVA was used and again showed no statistically significant difference between the three groups (*Bur, Piezo, Onlay*), due to the small sample size.

Please note that we were able to include an extra surgical sample (*Piezo, Collagen*) into our project and we were able to obtain a  $\mu$ CT scan for one of these extra rat leg samples (*Sample #500L*). See *Appendix*. The sample was segmented using our deep learning algorithm and was included in the statistical analysis – therefore the *Piezo, Collagen* group contained 4 samples ( $n=4$ ), whereas all other groups contained 3 samples ( $n=3$ ).

### 3.1.3 Porosity Analysis

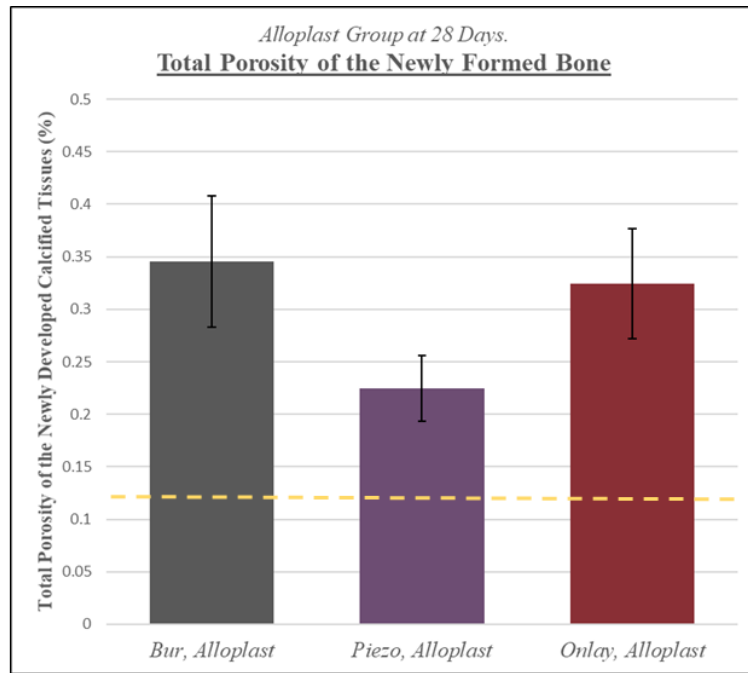


--- Control Average [Total Porosity of the cortical bone, %]

**Figure 32.** Total porosity data for the xenograft group at day 28.

This figure compares the data containing the total porosities of the new bone formation for the 28-day *Xenograft* samples.

For our “*qualitative*” analysis, the density of the newly formed bone was examined. In the 28-day *Xenograft* groups, selective cortical penetrations with a *Bur* yielded the highest total porosity of new bone formation. ANOVA was used and showed no statistically significant differences between the three groups (*Bur*, *Piezo*, *Onlay*). However, each experimental group displayed higher porosities relative to the *Control* group (yellow line in Figure 32).

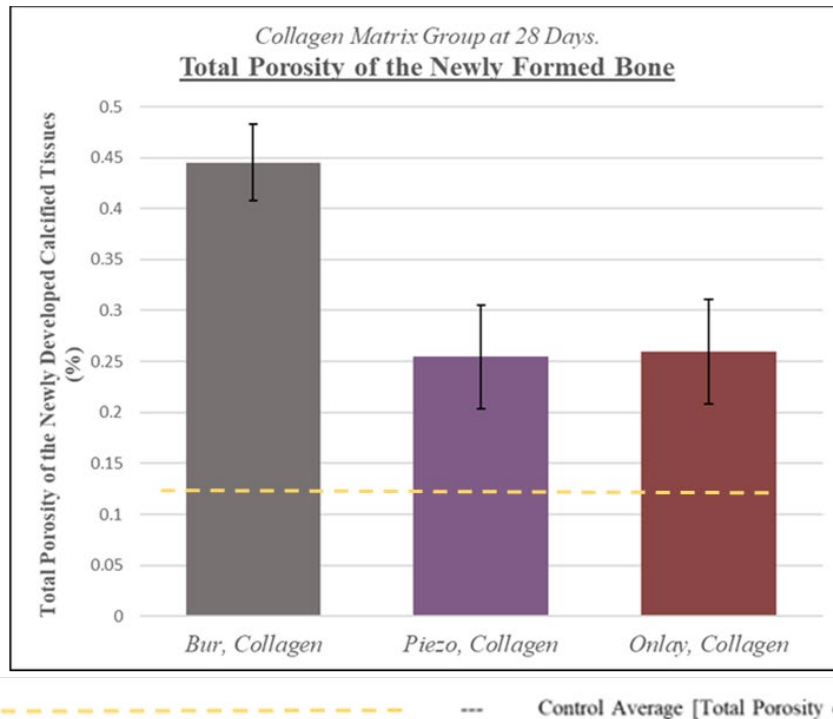


--- Control Average [Total Porosity of the cortical bone, %]

**Figure 33. Total porosity data for the alloplast group at day 28.**

This figure compares the data containing the total porosities of the new bone formation for the 28-day *Alloplast* samples.

For our qualitative analysis of the density of the newly formed bone in the 28-day *Alloplast* groups, again, selective cortical penetrations with a *Bur* yielded the highest total porosity of new bone formation. ANOVA was used and showed no statistically significant differences between the three groups (*Bur*, *Piezo*, *Onlay*). However, each experimental group displayed higher porosities relative to the *Control* group (yellow line in Figure 33).



**Figure 34.** Total porosity data for the collagen matrix group at day 28.

This figure compares the data containing the total porosities of the new bone formation for the 28-day *Collagen* samples.

For our qualitative analysis of the density of the newly formed bone in the 28-day *Alloplast* groups, selective cortical penetrations with a *Bur* yielded the highest total porosity of new bone formation. ANOVA was used and showed no statistically significant differences between the three groups (*Bur, Piezo, Onlay*). Each experimental group displayed higher porosities relative to the *Control* group (yellow line in Figure 34).

Again, note that we were able to include an extra surgical sample (*Piezo, Collagen*) into our project and we were able to obtain a  $\mu$ CT scan for one of these extra rat leg sample (*Sample #500L*). The sample was segmented using our deep learning algorithm and was included in the statistical analysis – therefore the *Piezo, Collagen* group contained 4 samples ( $n=4$ ), whereas all other groups contained 3 samples ( $n=3$ ).

### 3.2 Hematological Analysis

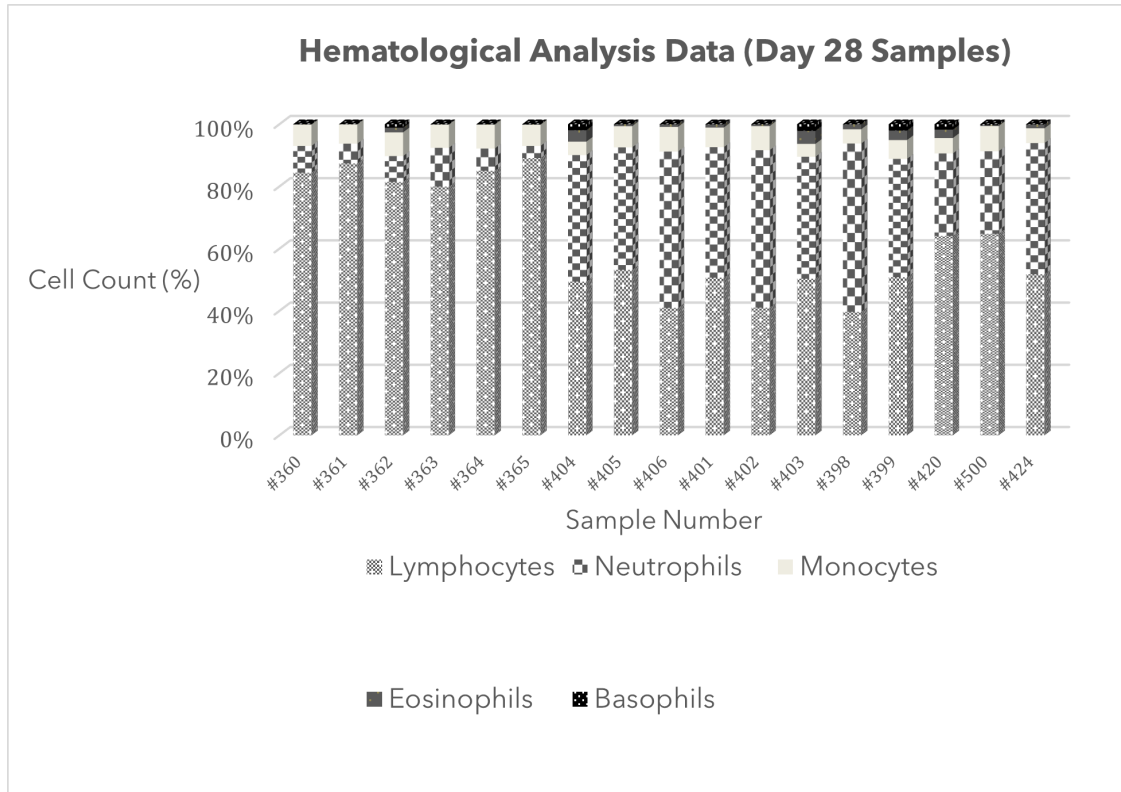


Figure 35. Differential white blood cell counts (%) at the time of sacrifice (day 28).

At day 28, no statistically significant differences were found in *lymphocyte* count, *neutrophil* count, *monocyte* count, *eosinophil* count, and *basophil* count when compared to the normal hematological range(s) in rodents.

### 3.3 Post-Operative Weight Monitoring Analysis

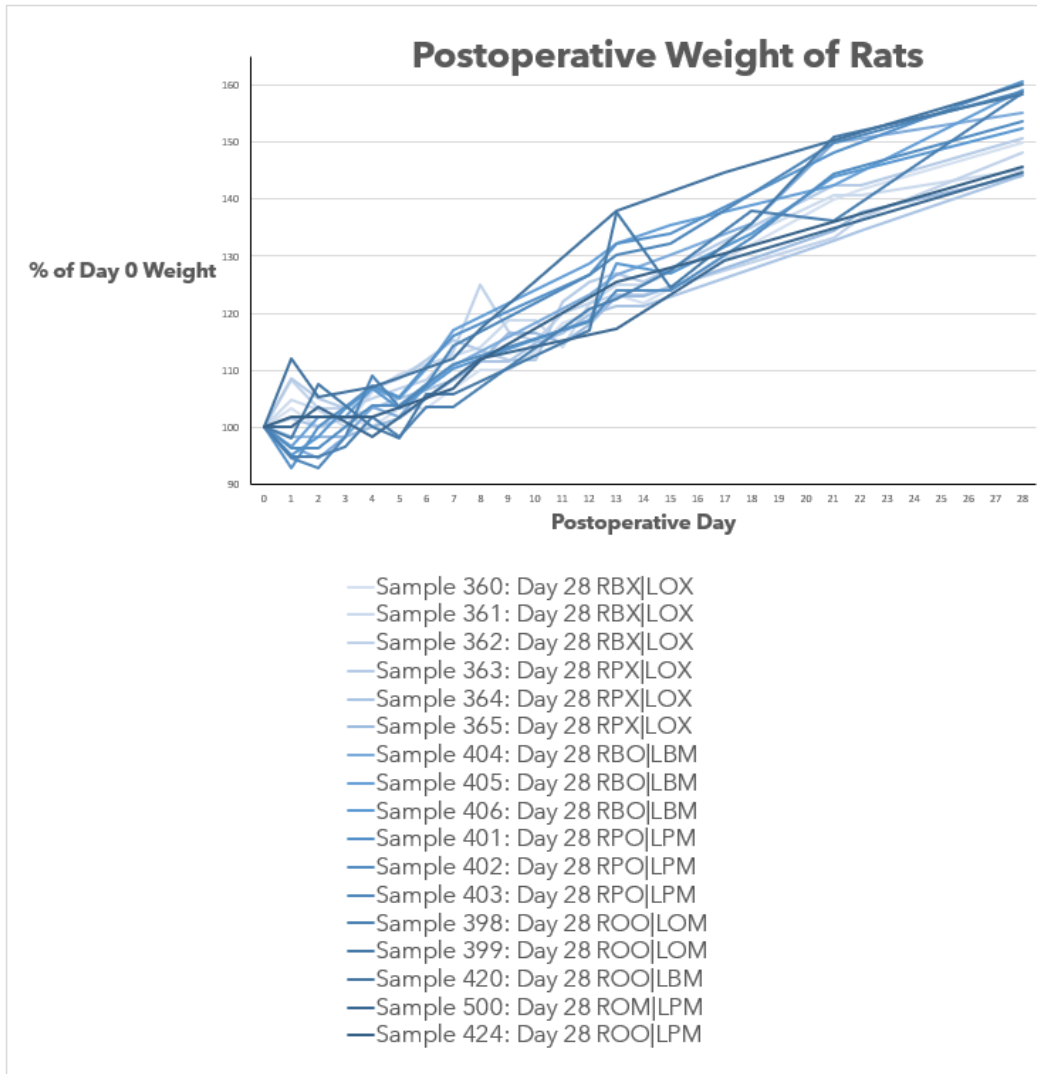


Figure 36. Graph of the post-operative weight in % of the original weight.

For the post-operative weights of the rats, the data was converted in % of the original weight and then ANOVA was calculated and there were no statistically significant differences between the samples.

## **CHAPTER 4**

### **DISCUSSION**

#### **4.1 Imaging Analysis**

The results from the present investigation show clear differences in hard tissue reaction and bone graft healing in response to the nine different surgical techniques tested, with or without the use of selective cortical penetrations. They also illustrate the power of microcomputed tomography and machine learning.

Sample preparation for histological processing is very challenging and when trying to isolate small regions of interest on the micron level, it can be difficult to keep the hard and soft tissues intact and sectioning may even lead to the inadvertent loss of valuable samples/data. Micro-computed tomography ( $\mu$ CT) is an increasingly useful tool for visualizing and analyzing three-dimensional structures.  $\mu$ CT scans typically contain 600-1,000 slices of data whereas histological sectioning may only yield one or two slices of interest. The 3D microscope (Xradia Versa 520, Zeiss) allowed us to obtain accurate, non-destructive images of our virtual samples and analyze thousands of slices of data at a very high resolution (10  $\mu$ m).

While our  $\mu$ CT scans provided us with tens of thousands slices of data, it was a challenge to determine the best way of extracting meaningful data from the large samples. The small voxel size of our scans allowed us to visualize the porosities and intricacies of the cortical and trabecular bone in the rat tibia, however traditional

segmentation techniques were not accurate enough to extract useful data. First, traditional window-leveling and thresholding techniques were attempted to separate the new bone formation from the original cortical bone, the bone graft particles, the background, and the noise in our volumes. The contrasts in our high resolution  $\mu$ CT scans were not distinct enough for these methods because there were highly radio-opaque bone graft particles, cortical bone, and new bone matrix. There were distinct peaks distinguishing background from the highly mineralized tissues, however these techniques were not able to separate the volumes of interest for this study. Manually segmenting each slice of data would have required too much time and manpower so we turned to machine learning. And our convolution neural network (CNN) algorithm was very successful in identifying the original tibial diaphysis anatomy (based on our training input), the newly formed hard tissues, and the isolated bone graft particles. Our deep neural network algorithm allowed us to extract the most possible data from our  $\mu$ CT scans than previously thought possible. And, the model is flexible enough to be modified based on the requirements of each individual study design – yielding unbiased, reliable and accurate segmentations in a valid and reproducible way with relatively little manual inputs.

Also, the choice to not use a membrane in our study made this experiment feasible because stabilizing a membrane over our grafts in a rat tibia is extremely technique sensitive. And the results from this study (and others) showed that new bone growth can be achieved without the use of a cell occlusive membrane. When preparing our surgical sites, special care was taken to keep the periosteum intact, which allowed for

bone graft material stabilization under periosteum only and subsequent hard tissue formation in the graft site(s).

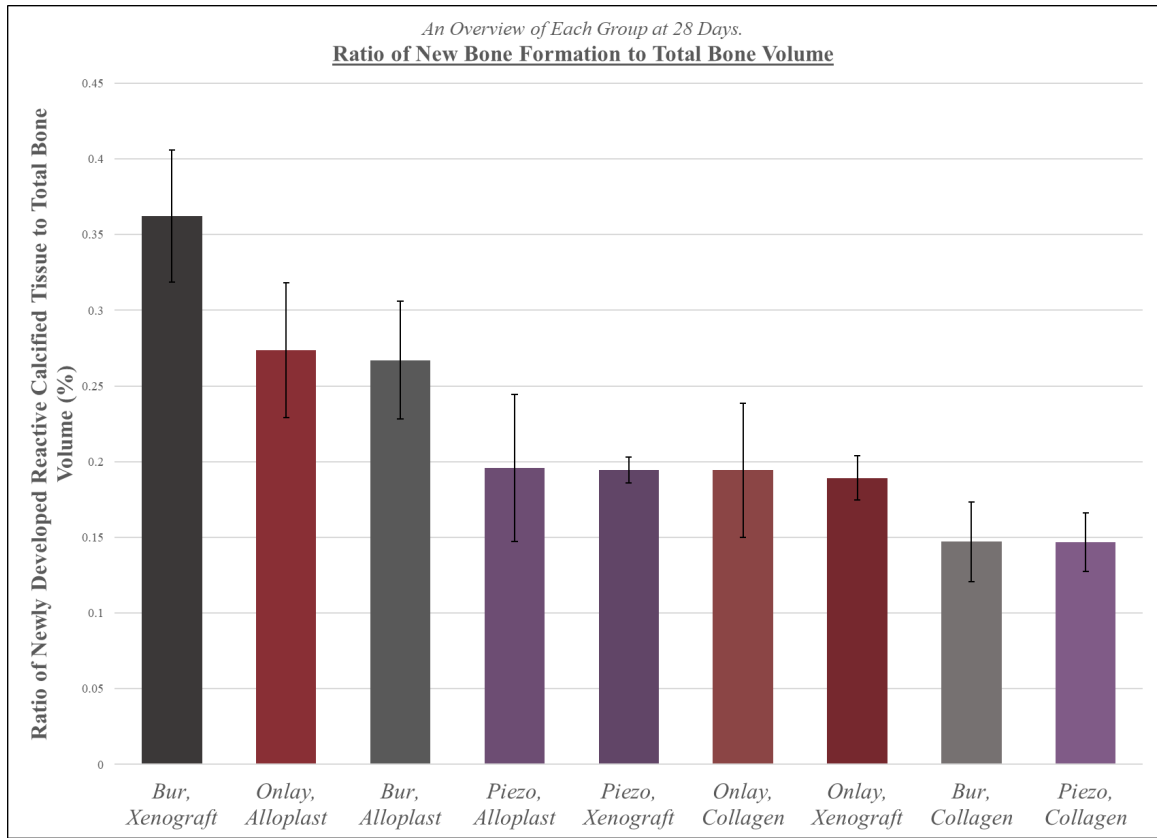
#### *4.1.1 New Bone Formation*

As mentioned previously, selective cortical penetrations are thought to increase the blood supply to the bone graft site(s), enhancing angiogenesis, bone healing, and integration of bone graft. However, the benefits of such techniques have not yet been proven in longitudinal human clinical trials. This study confirmed that selective cortical penetrations are not entirely necessary for new bone formation to occur (*Onlay* group). The SCP groups (*Bur, Piezo*) also yielded new bone formation.

The sample size in this study ( $n=3$ ) was small and this was one of the limitations of this study. The resulting ratios of new bone formation to total bone volume were quite variable and there were no statistically significant differences found between the experimental groups. There were also no clear patterns in found when comparing all nine bone grafting techniques (Figure 37) except that the collagen matrix groups tended to show lower levels of new bone formation, perhaps because the collagen matrix used did not maintain the graft site volume long enough to allow for new bone formation. Interestingly though, the collagen matrix groups did yield new bone gain. Whether this is due to the grafting material, or the recipient site preparation is unclear.

A study by Goldman *et al.* [94] attributed part of this new bone formation in the outer surface of the bone to the disturbance of the periosteum alone. A combination of both the elevation of a full-thickness flap and the surgical procedures damages the bone structure and an increased response of the tissues would be expected. A recent histomorphometric study by Kernitsky *et al.* [95] showed that in a rat tibia model, full thickness flap reflection with or without selective cortical penetrations will yield an increased thickness of cortical bone by day 28 due to a deposition of new bone on the outer surface.

The present research also demonstrated that both xenogenic and alloplastic bone substitutes can be used successfully for bone grafting surgery. Figures 26 and 27 show new bone formation with both bone grafting materials and hard tissue encapsulated bone graft particles are present in our  $\mu$ CT scans. Dense, radiopaque calcifications (new bone formation) surrounded and were in intimate contact with the two bone substitute materials. When assessing the “quantity” of the new bone formation, measurements revealed no superior grafting material, perhaps due to the small sample size, but when assessing the “quality” of the newly formed bone, selective cortical penetrations (SCPs) with a piezoelectric knife yielded some interesting results.



**Figure 37. Ratio of New Bone Formation to Total Bone Volume Overview.**

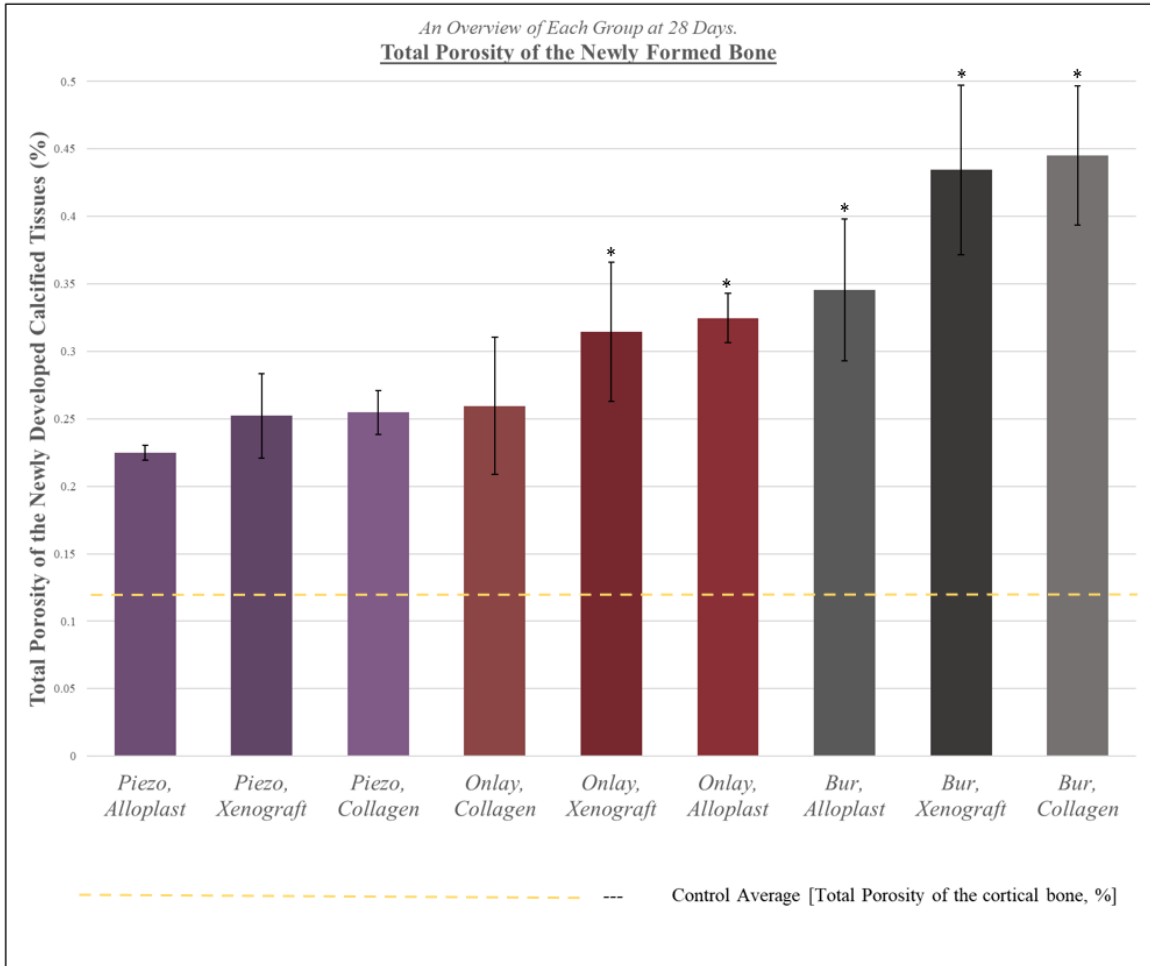
Figure 37 shows the new bone formation data for all 28-day samples in descending order. The *Bur, Xenograft* group yielded the highest ratio of new bone formation to total bone volume however there were no statistically significant differences between the groups. Each of the nine bone grafting techniques tested in this study did however result in new bone formation over the surgical site.

#### 4.1.2 Total Porosity of Newly Formed Bone

The main clinical advantage of the piezoelectric knife is that it can cut hard tissues and is generally safe to use adjacent to soft tissues. Although it does not cut the soft tissues, it is still possible to induce direct damage with increased pressure or damage from excessive overheating. Rashad *et al.*, however showed that the heat produced by rotary devices (*Bur*) is higher than that produced by ultrasonic devices (*Piezo*). [62] Additionally, Anesi *et al.* [96] observed that both techniques induced osteocyte death (RAP effect), but that the viable osteocytes were found significantly closer to the osteotomy edge of piezosurgery samples, compared to conventional rotary bur. Findings like this suggest that piezosurgery causes less damage to the bone and leads to a difference physiological response. Whole scans of our experimental samples showed that, for the *Piezo* and *Onlay* study groups, new bone formation was consistently found on one side of the tibial diaphysis (the side where the SCPs were made, and the graft was placed). Several, but not all, of our *Bur* samples however showed new bone formation not only above graft site, but also on the adjacent side of the tibial diaphysis (*see Figure 26 and Figure 27*). This “undesirable” bone growth could be attributed to increased hard tissue damage with a conventional bur.

The results of the present investigation showed that the porosities of the newly formed bone varied depending on which surgical technique was used. (Figure 38) The *Control* groups showed a uniformly dense cortical layer with little porosity and the surgical sites in which selective cortical penetrations were made with a piezoelectric

knife (*Piezo*) showed dense, newly formed bone that was *not* statistically different from than that of the *Control* group. This phenomenon seems to be triggered by the specific ultrasonic vibrations and also has been shown to lead to bone hypermineralization. [97] This could be useful in periodontal surgery for implant site preparation, site development, and influencing the secondary stability of dental implants.



**Figure 38. Total Porosity of the Newly Formed Bone Overview.**

Figure 38 shows the total porosity data for all 28-day samples in ascending order. The Dunnett’s test was used to compare total porosities to the control and unlike the other groups, the *Piezo* group was the only one that was consistently not statistically significant from the control. This result can be interpreted various ways, but it shows that the newly formed bone in sites in which SCPs are created with a piezoelectric knife is the most “cortical-like”.

## **4.2 Hematological Analysis**

No statistical differences were obtained in the white blood cell counts at day 28. This indicates that there were no hematological abnormalities throughout the post-operative healing period and that the effects of our bone grafting surgeries were not systemic.

## **4.3 Weight Analysis**

No statistically significant differences in post-operative weights were found between the different samples during the 28 days of post-operative healing.

Daily behavioral assessments during the first two-post operative weeks revealed no abnormalities in food consumption, ambulation (physical activity), distress and overall behavior supporting the conclusion that healing was uneventful.

## CHAPTER 5

### CONCLUSION

Within the limitations of this study (mainly the small number of subjects), it is possible to conclude that the piezoelectric knife is a valid alternative to conventional carbide burs when making selective cortical penetrations prior to bone grafting surgery. Although this is a preliminary study, we have shown that new bone formation can be obtained with the three tested grafting materials: a xenogenic bone graft, an alloplast, and even a collagen matrix. We have also shown that bone augmentation can be achieved without the use of a cell occlusive membrane or without the use of selective cortical penetrations. The newly formed bone over sites with SCPs created with a piezoelectric knife, however, showed statistically significant different total porosities making the new bone formation in the *Piezo* groups more “cortical-like” at 28 days. This study also illustrated that deep learning, and the use of convolutional neural networks is a powerful tool capable of extracting enormous amounts of data from  $\mu$ CT volumes. The feature detection capabilities of our algorithm can be adapted for future projects and the applications of this advanced innovative technology are virtually endless. Another limitation of this study was the lack of histology. Due to delays caused by the COVID-19 pandemic, histological analysis was not feasible for this project and future studies should be conducted with histology, larger sample sizes, and more time points. Our preliminary study, though, was a great success which hopefully helped shed some light on the potential applications of deep learning in research and the piezoelectric knife in bone grafting surgeries.

## APPENDIX

| 28 DAY XENOGRAFT GROUP |         |     | Volume of Reactive Bone Formation [ $\mu\text{m}^3$ ] | Volume of Original Tibia Cortex [ $\mu\text{m}^3$ ] | Volume of Graft [ $\mu\text{m}^3$ ] | Total Volume [ $\mu\text{m}^3$ ] | Total Volume of Bone (Excluding Graft) [ $\mu\text{m}^3$ ] | Ratio of Reactive Calcified Tissue to Total Bone |
|------------------------|---------|-----|---|---|-------------------------------------|----------------------------------|--|--|
| BUR                    | #360 R. | ROI | 15446251387   | 19974360773   | 6254039910                          | 41674652071                      | 35420612161  | 0.436080871                                      |
|                        | #361 R. | ROI | 12872152686   | 22345908519   | 8922772022                          | 44140833227                      | 35218061206  | 0.365498618                                      |
|                        | #362 R. | ROI | 8038332416  | 20183521285   | 8084899998                          | 36306753700                      | 28221853702  | 0.284826521                                      |
| PIEZO                  | #363 R. | ROI | 4771749742  | 22071400661   | 11319908057                         | 38163058460                      | 26843150403  | 0.177764147                                      |
|                        | #364 R. | ROI | 2,559,339,781.87                                      | 9,854,867,847.68                                    | 3650239779                          | 16064447409                      | 12414207630  | 0.206162154                                      |
|                        | #365 R. | ROI | 6392691085  | 25656315114   | 14707796497                         | 46756802696                      | 32049006199  | 0.199466125                                      |
| ONLAY                  | #361 L. | ROI | 11,460,795,125.93                                     | 54,385,177,876.23                                   | 21887436677                         | 87733409679                      | 65845973002  | 0.17405461                                       |
|                        | #364 L. | ROI | 8441419386  | 39663421740   | 20466719197                         | 68571560323                      | 48104841125  | 0.175479623                                      |
|                        | #365 L. | ROI | 12785652452   | 45756285445   | 18166228451                         | 76708166348                      | 58541937898  | 0.218401592                                      |

Figure 39. Volume Analysis Data for Day 28 Xenograft Samples.

| 28 DAY ALLOPLAST GROUP |         |     | Volume of Reactive Bone Formation [ $\mu\text{m}^3$ ] | Volume of Original Tibia Cortex [ $\mu\text{m}^3$ ] | Volume of Graft [ $\mu\text{m}^3$ ] | Total Volume [ $\mu\text{m}^3$ ] | Total Volume of Bone (Excluding Graft) [ $\mu\text{m}^3$ ] | Ratio of Reactive Calcified Tissue to Total Bone |
|------------------------|---------|-----|---|---|-------------------------------------|----------------------------------|--|--|
| BUR                    | #404 R. | ROI | 3905028489  | 16210264382   | 807210364.8                         | 20922503236                      | 20115292871  | 0.194132321                                      |
|                        | #405 R. | ROI | 2,587,429,113.64                                      | 6,648,682,545.34                                    | 114396597.3                         | 9350508256                       | 9236111659   | 0.280142684                                      |
|                        | #406 R. | ROI | 9766420734  | 20093926102   | 934338096.4                         | 30794684932                      | 29860346835  | 0.327069903                                      |
| PIEZO                  | #401 R. | ROI | 8562783208  | 20640963701   | 901465105.9                         | 30105212015                      | 29203746909  | 0.293208376                                      |
|                        | #402 R. | ROI | 2,305,207,812.36                                      | 12,948,909,125.66                                   | 552351040.1                         | 15806467978                      | 15254116938  | 0.151120371                                      |
|                        | #403 R. | ROI | 3,305,632,226.79                                      | 19,782,955,986.61                                   | 466960003.8                         | 23555548217                      | 23088588213  | 0.143171691                                      |
| ONLAY                  | #399 R. | ROI | 9401430785  | 35400748429   | 1942530989                          | 46744710203                      | 44802179214  | 0.209843158                                      |
|                        | #420 R. | ROI | 19545400280   | 34834845102   | 2823135838                          | 57203381220                      | 54380245382  | 0.359420965                                      |
|                        | #424 R. | ROI | 12174384725   | 36087639462   | 1261542301                          | 49523566489                      | 48262024187  | 0.252255991                                      |

Figure 40. Volume Analysis Data for Day 28 Alloplast Samples.

| 28 DAY COLLAGEN GROUP |         |     | Volume of Reactive Bone Formation [ $\mu\text{m}^3$ ] | Volume of Original Tibia Cortex [ $\mu\text{m}^3$ ] | Volume of Graft [ $\mu\text{m}^3$ ] | Total Volume [ $\mu\text{m}^3$ ] | Total Volume of Bone (Excluding Graft) [ $\mu\text{m}^3$ ] | Ratio of Reactive Calcified Tissue to Total Bone |
|-----------------------|---------|-----|---|---|-------------------------------------|----------------------------------|--|--|
| BUR                   | #404 L. | ROI | 4382605632  | 21828836231   | N/a                                 | 26211441862                      | 26211441862  | 0.167202005                                      |
|                       | #406 L. | ROI | 6486520253  | 29717077898   | N/a                                 | 36203598151                      | 36203598151  | 0.179167834                                      |
|                       | #420 L. | ROI | 2356519097  | 22409073997   | N/a                                 | 24765593094                      | 24765593094  | 0.095152944                                      |
| PIEZO                 | #401 L. | ROI | 2791413559  | 15614419104   | N/a                                 | 18405832663                      | 18405832663  | 0.151659184                                      |
|                       | #403 L. | ROI | 2196520433  | 18648415161   | N/a                                 | 20844935594                      | 20844935594  | 0.105374297                                      |
|                       | #424 L. | ROI | 6322339846  | 25731132550   | N/a                                 | 32053472396                      | 32053472396  | 0.197243524                                      |
|                       | #500 L. | ROI | 3652904741  | 23895694550   | N/a                                 | 27548599291                      | 27548599291  | 0.132598565                                      |
| ONLAY                 | #398 L. | ROI | 3956896617  | 33303614715   | N/a                                 | 37260511332                      | 37260511332  | 0.106195446                                      |
|                       | #399 L. | ROI | 8,643,093,279.82                                      | 26,130,907,687.51                                   | N/a                                 | 34774000967                      | 34774000967  | 0.248550441                                      |
|                       | #500 R. | ROI | 11849210620   | 40070975033   | N/a                                 | 51920185653                      | 51920185653  | 0.228219727                                      |

Figure 41. Volume Analysis Data for Day 28 Collagen Samples.

| 28 DAY XENOGRAFT GROUP | Sample  | Slice Number | Surface Area of New Bone [µm <sup>2</sup> ] | Zone (ROI) of New Bone [µm <sup>2</sup> ] | Density       | Mean Density | Total Porosity |
|------------------------|---------|--------------|---|---|---------------|--------------|----------------|
| BUR                    | #360 R. | 217          | 42,688,731.61                               | 78,066,091.14                             | 0.546828091   | 0.484805176  | 0.515194824    |
|                        |         | 271          | 46,495,551.46                               | 81,791,868.22                             | 0.568461786   |              |                |
|                        |         | 406          | 42,235,330.17                               | 92,883,776.01                             | 0.454711597   |              |                |
|                        | #361 R. | 509          | 51,753,474.97                               | 140,170,042.07                            | 0.36921923    | 0.550672085  | 0.449327915    |
|                        |         | 197          | 67,924,223.76                               | 121,080,660.14                            | 0.560983263   |              |                |
|                        |         | 251          | 62,249,328.38                               | 131,126,887.07                            | 0.474725892   |              |                |
|                        |         | 352          | 64,336,455.17                               | 117,783,759.62                            | 0.546225179   |              |                |
|                        | #362 R. | 450          | 48,967,936.19                               | 78,884,607.48                             | 0.620754007   | 0.661086849  | 0.338913151    |
|                        |         | 303          | 27,343,248.65                               | 37,835,661.37                             | 0.72268457    |              |                |
|                        |         | 377          | 30,380,846.36                               | 42,228,532.44                             | 0.719438839   |              |                |
|                        |         | 492          | 28,682,291.19                               | 49,624,104.96                             | 0.577991104   |              |                |
|                        | PIEZO   | #363 R.      | 259   | 9,767,797.16                              | 13,449,270.14 | 0.726269683  | 0.718448592    |
| 326                    |         |              | 9,091,456.06                                | 10,849,048.08                             | 0.837995739   |              |                |
| 467                    |         |              | 9,645,503.95                                | 16,320,177.77                             | 0.591017089   |              |                |
| 579                    |         |              | 11,319,578.66                               | 15,754,198.84                             | 0.718511857   |              |                |
| 38                     |         |              | 14,646,987.62                               | 19,097,594.53                             | 0.766954581   |              |                |
| #364 R.                |         | 178          | 15,131,984                                  | 20,617,304.34                             | 0.733945804   | 0.750450192  | 0.249549808    |
|                        |         |              |   |   |               |              |                |
| #365 R.                |         | 308          | 12,162,939.35                               | 16,077,158.87                             | 0.756535371   | 0.774537196  | 0.225462804    |
|                        |         | 446          | 14,496,222.49                               | 17,074,616.93                             | 0.848992545   |              |                |
|                        |         | 551          | 13,068,401.92                               | 17,484,185.94                             | 0.747441257   |              |                |
|                        |         | 676          | 18,848,365.79                               | 25,293,721.78                             | 0.745179612   |              |                |
|                        |         |              |   |   |               |              |                |
| ONLAY                  | #361 L. | 200          | 4,971,873.50                                | 5,707,403.74                              | 0.871127      | 0.721928239  | 0.278071761    |
|                        |         | 400          | 7,388,338.11                                | 8,854,540.40                              | 0.834412378   |              |                |
|                        |         | 600          | 24,643,663.79                               | 43,323,411.31                             | 0.568830179   |              |                |
|                        |         | 800          | 21,312,887.48                               | 34,748,702.76                             | 0.6133434     |              |                |
|                        | #364 L. | 200          | 3,239,019.84                                | 4,124,233.23                              | 0.785362917   | 0.664503091  | 0.335496909    |
|                        |         | 400          | 9,712,919.12                                | 13,687,684.61                             | 0.709610091   |              |                |
|                        |         | 600          | 16,411,227.05                               | 26,354,351.34                             | 0.622714133   |              |                |
|                        |         | 800          | 13,647,522.92                               | 21,257,978.53                             | 0.540325225   |              |                |
|                        | #365 L. | 200          | 9,120,610.28                                | 10,695,944.25                             | 0.8527167     | 0.670109794  | 0.329890206    |
|                        |         | 400          | 11,421,900.12                               | 16,922,449.32                             | 0.674955493   |              |                |
|                        |         | 600          | 13,014,727.00                               | 24,568,795.80                             | 0.529725881   |              |                |
|                        |         | 800          | 13,522,993.30                               | 21,704,817.27                             | 0.623041103   |              |                |
|                        |         |              |   |   |               |              |                |
|                        |         |              |   |   |               |              |                |
|                        |         |              |   |   |               |              |                |

Figure 42. Density Analysis Data for Day 28 Xenograft Samples.

| 28 DAY ALLOPLAST GROUP | Sample  | Slice Number | Surface Area of New Bone | Zone (ROI) of New Bone | Density     | Mean Density | Total Porosity |
|------------------------|---------|--------------|--------------------------|------------------------|-------------|--------------|----------------|
| BUR                    | #404 R. | 58           | 14,539,694.31            | 17,832,706.45          | 0.815338622 | 0.777898213  | 0.222101787    |
|                        |         | 160          | 13,489,867.28            | 16,071,375.55          | 0.839372289 |              |                |
|                        |         | 264          | 13,839,467.88            | 18,512,428.44          | 0.747577117 |              |                |
|                        |         | 356          | 13,536,002.25            | 19,083,476.93          | 0.709304824 |              |                |
|                        |         | 296          | 13,328,407.98            | 22,920,040.09          | 0.581517656 |              |                |
|                        |         | 350          | 8,673,898.58             | 15,364,439.30          | 0.564543776 |              |                |
|                        | #405 R. |              |                          |                        |             | 0.573030716  | 0.426969284    |
|                        | #406 R. | 304          | 25,558,686.49            | 41,559,836.34          | 0.614985254 | 0.61279826   | 0.38720174     |
|                        |         | 395          | 27,462,089.92            | 41,696,589.64          | 0.658617171 |              |                |
|                        |         | 496          | 23,457,750.51            | 37,748,977.53          | 0.621414196 |              |                |
|                        |         | 610          | 18,854,735.51            | 33,900,638.18          | 0.556176418 |              |                |
|                        |         |              |                          |                        |             |              |                |
|                        |         |              |                          |                        |             |              |                |
| PIEZO                  | #401 R. | 537          | 16,999,580.25            | 22,190,237.64          | 0.76608374  | 0.737918287  | 0.262081713    |
|                        |         | 640          | 17,626,755.14            | 24,840,786.09          | 0.709589265 |              |                |
|                        |         | 741          | 18,562,958.03            | 24,380,790.30          | 0.761376387 |              |                |
|                        |         | 847          | 22,949,129.38            | 32,113,583.04          | 0.714623758 |              |                |
|                        |         | 171          | 7,784,959.47             | 9,798,385.54           | 0.794514508 |              |                |
|                        |         | 284          | 4,588,537.55             | 6,437,778.67           | 0.712751678 |              |                |
|                        | #402 R. | 381          | 7,022,795.60             | 8,859,074.75           | 0.792723371 | 0.750579376  | 0.249420624    |
|                        |         | 492          | 9,541,738.53             | 13,585,873.32          | 0.702327948 |              |                |
|                        |         | 326          | 7,056,326.84             | 8,366,657.24           | 0.843386628 |              |                |
|                        |         | 416          | 4,375,875.24             | 4,882,576.87           | 0.896222498 |              |                |
|                        |         | 506          | 3,425,302.99             | 4,148,872.91           | 0.825598437 |              |                |
|                        |         | 614          | 5,332,527.91             | 6,810,069.85           | 0.783035714 |              |                |
| ONLAY                  | #399 R. | 200          | 12,753,644.04            | 16,175,748.63          | 0.788442274 | 0.740352748  | 0.259647252    |
|                        |         | 400          | 5,775,750.97             | 6,650,772.68           | 0.868433075 |              |                |
|                        |         | 600          | 8,122,308.04             | 11,354,014.40          | 0.715368834 |              |                |
|                        |         | 800          | 17,294,844.69            | 29,354,750.45          | 0.58916681  |              |                |
|                        |         | 150          | 28,222,140.49            | 48,305,405.45          | 0.584243942 |              |                |
|                        |         | 350          | 33,902,334.37            | 60,683,476.62          | 0.558674886 |              |                |
|                        | #420 R. | 550          | 32,257,453.89            | 57,521,574.02          | 0.56078879  | 0.57168753   | 0.42831247     |
|                        |         | 750          | 21,071,575.51            | 36,140,719.52          | 0.583042501 |              |                |
|                        |         | 275          | 14,892,586.00            | 23,478,736.58          | 0.634300996 |              |                |
|                        |         | 400          | 15,601,515.63            | 22,369,768.08          | 0.697437523 |              |                |
|                        |         | 525          | 15,003,989.23            | 21,127,115.72          | 0.710176885 |              |                |
|                        |         | 650          | 17,961,238.54            | 22,013,277.76          | 0.815927493 |              |                |

Figure 43. Density Analysis Data for Day 28 Alloplast Samples.

| 28 DAY COLLAGEN GROUP | Sample  | Slice Number | Surface Area of New Bone | Zone (ROI) of New Bone | Density    | Mean Density | Total Porosity |
|-----------------------|---------|--------------|--------------------------|------------------------|------------|--------------|----------------|
| BUR                   | #404 L. | 275          | 5,350,988.64             | 8,110,598.88           | 0.65975259 | 0.624970279  | 0.375029721    |
|                       |         | 384          | 6,885,344.09             | 10,606,713.47          | 0.64914963 |              |                |
|                       |         | 537          | 8,457,197.01             | 16,381,322.04          | 0.51627073 |              |                |
|                       | #406 L. | 644          | 8,786,566.57             | 13,022,765.91          | 0.67470817 | 0.541338612  | 0.458661388    |
|                       |         | 247          | 10,246,558.30            | 16,628,746.64          | 0.61619547 |              |                |
|                       |         | 396          | 6,897,910.50             | 12,378,344.97          | 0.55725628 |              |                |
|                       |         | 526          | 5,967,786.85             | 11,703,549.38          | 0.50991256 |              |                |
|                       |         | 667          | 7,822,968.12             | 16,230,556.45          | 0.48199014 |              |                |
|                       | #420 L. | 270          | 4,977,644.91             | 10,213,028.00          | 0.48738189 | 0.497968924  | 0.502031076    |
|                       |         | 387          | 5,967,677.11             | 11,378,346.59          | 0.52447665 |              |                |
|                       |         | 512          | 7,906,210.23             | 15,303,051.64          | 0.51664272 |              |                |
|                       |         | 638          | 8,584,757.90             | 18,526,611.13          | 0.46337443 |              |                |
| PIEZO                 | #401 L. | 38           | 6,119,647.23             | 7,613,873.75           | 0.8037495  | 0.765818294  | 0.234181706    |
|                       |         | 144          | 5,897,942.89             | 7,553,132.83           | 0.78086048 |              |                |
|                       |         | 237          | 4,338,926.06             | 6,077,128.59           | 0.71397635 |              |                |
|                       |         | 332          | 6,159,128.82             | 8,054,445.79           | 0.76468686 |              |                |
|                       | #403 L. | 166          | 3,508,567.94             | 3,768,724.30           | 0.93096965 | 0.872844836  | 0.127155164    |
|                       |         | 256          | 4,066,335.09             | 5,065,457.00           | 0.80275779 |              |                |
|                       |         | 355          | 3,432,646.82             | 3,915,505.13           | 0.87668046 |              |                |
|                       |         | 460          | 3,341,541.48             | 3,793,019.06           | 0.88097144 |              |                |
|                       |         | 274          | 15,512,043.09            | 24,879,651.21          | 0.62348314 |              |                |
|                       | #424 L. | 392          | 17,114,252.05            | 25,425,536.57          | 0.67311272 | 0.629752382  | 0.370247618    |
|                       |         | 503          | 13,797,416.19            | 21,360,031.22          | 0.64594551 |              |                |
|                       |         | 605          | 14,198,704.08            | 24,630,508.73          | 0.57646816 |              |                |
|                       |         | 284          | 10,620,778.61            | 16,183,850.69          | 0.65625782 |              |                |
|                       | #500 L. | 399          | 7,833,672.41             | 9,837,917.75           | 0.79627342 | 0.712798813  | 0.287201187    |
|                       |         | 483          | 5,586,365.48             | 7,704,039.56           | 0.7251216  |              |                |
| 591                   |         | 3,240,821.16 | 4,811,606.67             | 0.67354241             |            |              |                |
| 100                   |         | 5,595,524.66 | 6,663,731.40             | 0.83969841             |            |              |                |
| ONLAY                 | #398 L. | 300          | 3,732,170.17             | 4,311,055.21           | 0.8657208  | 0.834684452  | 0.165315548    |
|                       |         | 500          | 10,722,480.11            | 13,448,700.98          | 0.79728742 |              |                |
|                       |         | 700          | 6,092,492.01             | 7,287,398.11           | 0.83603118 |              |                |
|                       |         | 200          | 20,944,319.90            | 25,835,924.49          | 0.81066655 |              |                |
|                       | #399 L. | 400          | 23,109,525.49            | 32,788,748.70          | 0.70480047 | 0.729534427  | 0.270465573    |
|                       |         | 600          | 20,183,460.84            | 26,387,285.48          | 0.76489341 |              |                |
|                       |         | 800          | 14,133,692.95            | 22,160,860.26          | 0.63777727 |              |                |
|                       | #500 R. | 100          | 31,914,922.70            | 39,191,739.72          | 0.81432779 | 0.657221453  | 0.342778547    |
|                       |         | 300          | 20,156,793.28            | 29,423,167.21          | 0.68506538 |              |                |
|                       |         | 500          | 14,107,122.80            | 23,731,920.72          | 0.59443662 |              |                |
|                       |         | 700          | 15,079,120.07            | 28,182,320.14          | 0.53505602 |              |                |

Figure 44. Density Analysis Data for Day 28 Collagen Samples.

| 28 DAY CONTROL GROUP | Sample  | Slice Number | Surface Area of Original Bone | Zone (ROI) of Cortical Bone | Density     | Mean Density | Total Porosity |
|----------------------|---------|--------------|-------------------------------|-----------------------------|-------------|--------------|----------------|
| CONTROL              | #359 L. | 100          | 38,890,346.63                 | 41,670,556.40               | 0.933281194 | 0.876409375  | 0.123590625    |
|                      |         | 300          | 36,872,196.99                 | 41,262,114.10               | 0.893609011 |              |                |
|                      |         | 500          | 33,730,450.30                 | 40,860,981.73               | 0.8254929   |              |                |
|                      |         | 700          | 35,469,908.89                 | 40,777,222.12               | 0.869846131 |              |                |
|                      |         | 900          | 35,470,822.63                 | 41,253,890.43               | 0.859817638 |              |                |
|                      | #355 R. | 100          | 27,432,390.83                 | 31,120,068.45               | 0.881501622 | 0.878182747  | 0.121817253    |
|                      |         | 300          | 28,778,685.84                 | 31,268,679.52               | 0.920367802 |              |                |
|                      |         | 500          | 26,425,475.00                 | 30,875,618.40               | 0.855868688 |              |                |
|                      |         | 700          | 26,891,021.93                 | 31,175,266.85               | 0.862575517 |              |                |
|                      | #395 L. | 900          | 28,265,219.42                 | 32,466,363.45               | 0.870600105 | 0.894194689  | 0.105805311    |
|                      |         | 100          | 33,654,877.72                 | 38,959,602.93               | 0.863840368 |              |                |
|                      |         | 300          | 33,826,685.75                 | 38,502,971.31               | 0.878547411 |              |                |
|                      |         | 500          | 34,527,930.58                 | 36,953,652.80               | 0.934357715 |              |                |
|                      |         | 700          | 32,969,168.73                 | 36,631,055.81               | 0.900033264 |              |                |

Figure 45. Density Analysis Data for Day 28 Control Samples.

| Sample Group                              | Sample Number  | Sample Label   | Neutrophils (%) | Lymphocytes (%) | Monocytes (%) | Eosinophils (%) | Basophils (%) |
|---|----------------|----------------|-----------------|-----------------|---------------|-----------------|---------------|
| Day 28 Samples:<br>Xenograft              | 360            | Day 28 RBX LOX | 8.59            | 84.43           | 6.86          | 0.09            | 0.02          |
|   | 361            | Day 28 RBX LOX | 6.36            | 87.41           | 6.19          | 0               | 0.05          |
|   | 362            | Day 28 RBX LOX | 8.31            | 81.45           | 7.64          | 1.5             | 1.1           |
|   | 363            | Day 28 RPX LOX | 12.47           | 79.96           | 7.45          | 0.06            | 0.06          |
|   | 364            | Day 28 RPX LOX | 7.13            | 85.05           | 7.67          | 0.14            | 0             |
|   | 365            | Day 28 RPX LOX | 3.91            | 89.13           | 6.82          | 0.1             | 0.05          |
| Day 28 Samples:<br>Alloplast and Collagen | 404            | Day 28 RBO LBM | 40.67           | 49.41           | 4.31          | 3.68            | 1.92          |
|   | 405            | Day 28 RBO LBM | 39.38           | 53.19           | 6.81          | 0.57            | 0.05          |
|   | 406            | Day 28 RBO LBM | 50.28           | 40.96           | 7.92          | 0.79            | 0.06          |
|   | 401            | Day 28 RPO LPM | 41.93           | 50.7            | 6.32          | 0.96            | 0.1           |
|   | 402            | Day 28 RPO LPM | 50.63           | 41.04           | 7.73          | 0.51            | 0.09          |
|   | 403            | Day 28 RPO LPM | 39.21           | 50.36           | 4.14          | 4.17            | 2.14          |
|   | 398            | Day 28 ROO LOM | 54.14           | 39.68           | 4.57          | 1.56            | 0.05          |
|   | 399            | Day 28 ROO LOM | 38.07           | 50.8            | 6.04          | 3.05            | 2.04          |
|   | 420            | Day 28 ROO LBM | 26.55           | 64.07           | 4.95          | 2.7             | 1.73          |
|   | 500            | Day 28 ROM LPM | 26.54           | 64.79           | 8.1           | 0.3             | 0.28          |
| 424                                       | Day 28 ROO LPM | 42.17          | 51.78           | 4.75            | 1.18          | 0.12            |               |

Figure 46. Hematology Data for Day 28 Experimental Samples.

| Sample Description | Day 28 Samples: Xenograft (Weight, g) |                         |                         |                         |                         |                         | Day 28 Samples: Alloplast and Collagen (Weight, g) |                         |                         |                         |                         |                         |                         |                         |                         |                         |                         |  |
|--------------------|---------------------------------------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|--|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|--|
|                    | 360<br>Day 28<br>RBXLOX               | 361<br>Day 28<br>RBXLOX | 362<br>Day 28<br>RBXLOX | 363<br>Day 28<br>RPXLOX | 364<br>Day 28<br>RPXLOX | 365<br>Day 28<br>RPXLOX | 404<br>Day 28<br>RBOLEB                            | 405<br>Day 28<br>RBOLEB | 406<br>Day 28<br>RBOLEB | 401<br>Day 28<br>RPOLEB | 402<br>Day 28<br>RPOLEB | 403<br>Day 28<br>RPOLEB | 398<br>Day 28<br>ROOLOM | 399<br>Day 28<br>ROOLOM | 420<br>Day 28<br>ROOLEB | 500<br>Day 28<br>ROMLPM | 424<br>Day 28<br>ROOLEP |  |
| Day 0              | 300                                   | 320                     | 300                     | 295                     | 305                     | 305                     | 280  | 295                     | 280                     | 270                     | 280                     | 290                     | 265                     | 290                     | 290                     | 295                     |                         |  |
| Day 1              | 310                                   | 335                     | 325                     | 320                     | 310                     | 300                     | 270  | 285                     | 280                     | 260                     | 265                     | 275                     | 260                     | 325                     | 290                     | 300                     |                         |  |
| Day 2              | 300                                   | 330                     | 310                     | 310                     | 305                     | 300                     | 255  | 300                     | 280                     | 260                     | 260                     | 275                     | 285                     | 305                     | 300                     | 300                     |                         |  |
| Day 3              | 305                                   | 320                     | 310                     | 305                     | 310                     | 300                     | 275  | 300                     | 290                     | 270                     | 275                     | 280                     | 275                     | 305                     | 300                     | 300                     |                         |  |
| Day 4              | 300                                   | 340                     | 315                     | 315                     | 315                     | 305                     | 290  | 315                     | 300                     | 280                     | 305                     | 295                     | 265                     | 310                     | 285                     | 300                     |                         |  |
| Day 5              | 310                                   | 350                     | 320                     | 320                     | 320                     | 310                     | 285  | 310                     | 295                     | 280                     | 290                     | 285                     | 260                     |                         |                         |                         |                         |  |
| Day 6              | 310                                   | 355                     | 325                     | 330                     | 325                     | 320                     | 300  | 325                     | 310                     | 290                     | 300                     | 300                     | 280                     |                         |                         |                         |                         |  |
| Day 7              | 320                                   | 360                     | 340                     | 340                     | 330                     | 330                     | 310  | 345                     | 325                     | 300                     | 320                     | 300                     | 280                     | 325                     | 315                     | 315                     |                         |  |
| Day 8              | 330                                   | 365                     | 375                     | 335                     | 340                     | 340                     |  |                         |                         |                         |                         |                         |                         | 340                     | 325                     | 330                     |                         |  |
| Day 9              | 330                                   | 380                     | 350                     | 330                     | 340                     | 355                     |  |                         |                         |                         |                         |                         |                         |                         |                         |                         |                         |  |
| Day 10             | 340                                   | 380                     | 345                     | 330                     | 350                     | 355                     |  |                         |                         |                         |                         |                         |                         |                         |                         |                         |                         |  |
| Day 11             | 355                                   | 365                     | 360                     | 360                     | 355                     | 350                     | 345  | 380                     | 355                     | 320                     | 355                     | 350                     | 310                     |                         |                         |                         |                         |  |
| Day 12             | 360                                   | 390                     | 365                     | 370                     | 365                     | 360                     | 355  | 390                     | 370                     | 335                     | 365                     | 355                     | 365                     | 400                     | 340                     | 370                     |                         |  |
| Day 13             | 370                                   | 400                     | 370                     | 375                     | 370                     | 375                     | 355  | 380                     | 370                     | 335                     | 365                     | 355                     | 365                     |                         |                         |                         |                         |  |
| Day 14             | 365                                   | 400                     | 370                     | 370                     | 370                     | 375                     | 365  | 400                     | 375                     | 335                     | 370                     | 370                     | 330                     |                         |                         |                         |                         |  |
| Day 15             |                                       |                         |                         |                         |                         |                         |  |                         |                         |                         |                         |                         |                         |                         |                         |                         |                         |  |
| Day 16             |                                       |                         |                         |                         |                         |                         |  |                         |                         |                         |                         |                         |                         |                         |                         |                         |                         |  |
| Day 17             |                                       |                         |                         |                         |                         |                         | 380  | 410                     | 395                     | 360                     | 395                     | 400                     | 360                     | 420                     | 375                     | 385                     |                         |  |
| Day 18             |                                       |                         |                         |                         |                         |                         |  |                         |                         |                         |                         |                         |                         |                         |                         |                         |                         |  |
| Day 19             |                                       |                         |                         |                         |                         |                         |  |                         |                         |                         |                         |                         |                         |                         |                         |                         |                         |  |
| Day 20             |                                       |                         |                         |                         |                         |                         |  |                         |                         |                         |                         |                         |                         |                         |                         |                         |                         |  |
| Day 21             | 420                                   | 450                     | 400                     | 420                     | 405                     | 410                     | 420  | 425                     | 415                     | 390                     | 420                     | 395                     | 400                     |                         |                         |                         |                         |  |
| Day 22             | 425                                   | 450                     | 410                     | 420                     | 410                     | 420                     |  |                         |                         |                         |                         |                         |                         |                         |                         |                         |                         |  |
| Day 23             |                                       |                         |                         |                         |                         |                         |  |                         |                         |                         |                         |                         |                         |                         |                         |                         |                         |  |
| Day 24             |                                       |                         |                         |                         |                         |                         |  |                         |                         |                         |                         |                         |                         |                         |                         |                         |                         |  |
| Day 25             |                                       |                         |                         |                         |                         |                         |  |                         |                         |                         |                         |                         |                         |                         |                         |                         |                         |  |
| Day 26             |                                       |                         |                         |                         |                         |                         |  |                         |                         |                         |                         |                         |                         |                         |                         |                         |                         |  |
| Day 27             |                                       |                         |                         |                         |                         |                         | 435  | 470                     | 450                     | 415                     | 445                     | 460                     | 420                     | 465                     | 420                     | 430                     |                         |  |
| Day 28             | 450                                   | 465                     | 445                     | 445                     | 440                     | 440                     |  |                         |                         |                         |                         |                         |                         |                         |                         |                         |                         |  |

Figure 47. Weight Data for Day 28 Experimental Samples.

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## CURRICULUM VITAE

