BONE MARROW REGENERATION FOLLOWING TIBIAL MARROW ABLATION IN RATS IS AGE DEPENDEDNT

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BONE MARROW REGENERATION FOLLOWING TIBIAL

MARROW ABLATION IN RATS IS AGE DEPENDEDNT

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To my parents, Simon and Susie Fisher, for their great love and support

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SUMMARY

Injuries to the marrow cavity result in rapid endosteal bone formation followed by bone remodeling and regeneration of the marrow. It is not known whether this process is affected by age, although the quality of marrow is markedly different in young and old animals. Whereas young animals have red marrow and comparatively high levels of mesenchymal stem cells, old animals have yellow marrow characterized by increased levels of fat cells and they have fewer mesenchymal stem cells. To test if marrow restoration differs as a function of age, we used the rat tibial bone marrow ablation model, which has been used to examine calcification during osteogenesis, effects of metal implants on osteointegration and remodeling of bone graft substitutes during marrow cavity restoration. These previous studies were conducted in 3-month old immunocompetent rats but analysis of many biomaterials requires the use of immune deficient animals; however, it is not known whether this will affect the healing process. Accordingly, we assessed bone marrow healing in nude rats aged 1 month, 3-months and 10-months using micro-CT and histomorphometry, and compared the results to our previous work using Sabra strain rats. Thus, we determined if restoration of bone marrow is age dependent; if differences in healing can be detected by micro-CT; if the quality of marrow differs in young and old rats; and if the time course of healing in 3-month immunocompromised animals is comparable to that seen in normal rats of the same age. After determining that there was a difference in the kinetics of the healing of aged rats we tested a new biomaterial which is suppose to induce the healing in aged rats. Porcine Bone Marrow Matrix (PBMM), is a material produced by decellularizing porcine bone marrow matrix. Boston Scientific provided it to us in a sterile liquid suspension. We

hypothesized that PBMM would provide a growth factor enriched scaffold that would enhance repopulation of the marrow cavity with multipotent stem cells.

Methods: Marrow was ablated in the left tibia of seven rats (rNu/rNu) per time point. At 0, 7, 14, 21, 28, 35 and 42 days post-surgery, the treated tibia and the contralateral tibia were harvested, fixed in 70% ethanol for 24 h and post-fixed in buffered formalin. Both tibias were scanned using microCT and trabecular BV/TV calculated. Mid-sagittal sections of decalcified paraffin embedded bones were stained with haematoxylin and eosin. BV/TV was calculated using ImagePro. Left tibias from untreated animals were used as controls for histology. Using this software we were also able to determine fat cell number per marrow cavity. Pilot study using PBMM was also conducted in aged rats and was part of the aged group study. From this pilot study we chose one time point to test the PBMM as an enhancer of bone marrow restoration. For this study we used five different groups testing the PBMM in a dose dependent study. As mention above, left tibia was treated while the right served as a control and was not treated.

Results: Micro-CT analysis for the 1-month animals showed high increase in bone formation and on day 21 the marrow was restored. High increase in bone formation was also noticed in 3-month animals on days 7 and 14, however their bone formation was significantly lower compared to 1-month old animals. By day 21 remodeling had reduced the area of trabecular bone by 50%. 10-month animals had less trabecular bone at days 7 and 14, the levels were sustained through 21 days. Histomorphometry indicated that bone formation peaked at day 7 in 1-month old rats with remodeling underway by day 14, as noted previously for Sabra strain rats. For 3-month old rats bone formation

peaked at day 7 as well, but restoration occurred only on day 21. However, in 10-month rats, peak bone formation occurred on day 14, with remodeling on day 28. In the pilot study of PBMM we got promising results for enhanced bone marrow restoration as we saw a significant decrease in trabecular bone when compare to day 42 control (No PBMM). However, when we proceed to the next study did not see this effect again. PBMM had an effect on the marrow cavity but it was not significantly different than with our control group.

Discussion: The significance of this study is in the development of a model that enables us to view bone marrow restoration in aged rats. Using this model we can examine various materials such as implants and bone grafts to learn more about bone recovery processes in general and also to test materials that might induce healing in older populations, in particular. As science advances so does human life expectancy. The aged population around the world is growing; in order to guaranty quality of life one will need to fully understand bone healing in elderly populations.

Conclusions: Endosteal bone formation and remodeling in 3-month nude rats is comparable to 3-month immunocompetent rats. Aged animals produce less primary bone that younger animals and remodeling is initiated later. Differences in micro-CT and histomorphometric analyses may reflect a reduction in calcification of the osteoid in the 10-month old animals. PBMM des not appear to enhance marrow restoration in aged rats.

CHAPTER 1

INTRODUCTION

Injuries to Bone Marrow

Injuries to the bone marrow initiate endosteal bone formation. This process is characterized by primary bone formation, resorption and marrow restoration (Boyan, 1993). The bone building cells that conduct primary bone formation are the osteoblasts. These cells derive from mesenchymal cells that are located at both the periosteum and the endosteum. They lay down osteoid, which is the organic matter of the matrix that eventually becomes calcified (Schwartz, 1995). Bone resorbing cells called osteoclasts are then recruited through osteoblast secretion of growth factors. They remodel the newly formed trabecular bone, leading to marrow tissue restoration. Pro-inflammatory mediators at the fracture site are also play an important role for signaling those cells to proliferate and differentiate. Cytokines such as IL-1, TNF-alpha and IL-6 promote osteoclast function. In contrast, Th2 cytokines such as IL-4 and IL-13 suppresses osteoclastogenesis (Datta, Ng *et al.*, 2008; Mountziaris and Mikos, 2008).

The bone healing process includes a replacement of the marrow tissue and the trabecular bone within it, to a new tissue. Injury to the marrow actually involves two processes: bone healing of the cortical bone and marrow tissue restoration. Therefore it is important to understand the consequences of both processes in order to better assess their effect in clinical applications. Any procedure that occurs today and involves either dental implant or joint replacement will involve restoration of the lost marrow and healing around the implant.

Bone Marrow Ablation Model

Bone marrow ablation mimics the process of injury to the marrow. In this model part of the marrow in the tibial cavity is removed initiating the endosteal bone formation (Bab, 1995). Age is known to be an important physiological factor that affects the healing process (Schwartz and Boyan, 1994). This model is well suited to study the differences in the healing process by looking at restoration of the marrow. By comparing different age groups it can be learned whether bone marrow ablation can induce systemic effects (Schwartz, Sela *et al.*, 1989; Gazit, Karmish *et al.*, 1990). This model was used previously to study various processes, for example: calcification and changes in extracelullar matrix vesicles during healing of the bone; the study hypothesis being that matrix vesicles play an important role as an initiation site for crystal formation. Indeed a change was found in matrix vesicles during the healing process that took place on day six in the vicinity of the vesicles closest to the calcification front. However, by day 21 they had lost their enzymatic activity and were comparable to those on day 0, indicating that once crystals have been formed their function ends (Schwartz, Sela *et al.*, 1989; Marshall, Schwartz *et al.*, 1991). Another study examined primary bone formation around dental and orthopedic implants: when the marrow is removed from the rat it mimics the process of ablation after joint replacement and new primary bone fills up the cavity. The ability of cells to act fast and to synthesize and mineralize osteoid is critical for long-term acceptance of implant materials (Schwartz, Amir *et al.*, 1991; Kohavi, Schwartz *et al.*, 1992; Schwartz, Braun *et al.*, 1993; Sela, Gross *et al.*, 2000).

In an additional study bone remodeling with or without bone substitutes was examined. Most of the available bone substitutes have this quality, but an additional

desirable character would be for the materials to resorb as new bone forms. By using bone marrow ablation models researchers are able to detect differences in bone formation, remodeling and marrow regeneration due to different bone grafts (Schwartz *et al.*, 2008). Bone marrow ablation was also adopted by other groups with some variation in the procedure to study the role of insulin-like growth factor (Tanaka, Barnes *et al.*, 1996); to study the effect of glucorticoid on marrow generation (Kondo, Tokunaga *et al.*, 2006); for characterizing the transcription factor Runx2/Cbfa1 (Tsuji, Komori *et al.*, 2004), and to learn about patterns of gene expression during imtramembranous bone regeneration (Kuroda, Virdi *et al.*, 2005).

Bone Marrow

The bone marrow is a connective tissue consisting of two components: the hematopoeta and the stroma. The first gives rise to red blood cells and precursors of white blood cells. The second is composed of mixtures of pluripotent cells from the mesodermal tissue, which today is termed marrow stromal cells (MSCs). Those cells differentiate to different cell lineage such as osteoblasts, chondrocytes and adipocytes (Black and Woodbury, 2001) . There are two types of marrow tissue: red marrow, which consists of a majority of hematopoietic cells and yellow marrow, which consists of mainly fat cells (Blebea, Houseni *et al.*, 2007; Fan, Hernandez-Pampaloni *et al.*, 2007). The ratio between the two different marrows alters with age with a normal physiological progressive conversion of red to yellow marrow (Ricci, Cova *et al.*, 1990). A normal development of bone cannot occur with the stroma compartment alone. Osteoclasts develop from the hematopoietic precursor cells and they have an essential part in braking down the bone and recycling the calcium in the body. Moreover, in vitro studies have

shown that there is no MSC differentiation without having both compartments working together (Boyle, 2003; Datta, Ng *et al.*, 2008). The MSCs are responsible in time of injury to proliferate and differentiate to new bone building cells. Consequently, if those cells, for some reason lose their ability to differentiate, healing might stall or stop. Many researchers are trying to find out the role of the adipose tissue as well. For a long time the tissue was considered to have no role other than filling out the empty spaces as the bone density in older people decreases with age. The role of the adipose cells is being investigated and researchers are trying to identify other functions for those cells (Gimble, Robinson *et al.*, 1996; Gimble and Nuttall, 2004).

Aging and Its Impact on Bone Healing

Some important questions concerning aging still remain without any clear answer. First, what is the effect of aging on bone marrow stem cells? Second, how important and to what extent do stem cells contribute to tissue repair (Rando, 2006) With aging there is regular conversion from red to yellow marrow. In the long bones, the replacement starts first in the diaphysis with relative preservation of metaphyseal hematopoietic tissue (Ricci, Cova *et al.*, 1990). Aging is also associated with decreasing bone formation and bone mass (Xiao, Fu *et al.*, 2007), delay in fracture healing (Bak and Andreassen, 1989; Fan, Crawford *et al.*, 2008) and decline of regenerative properties (Conboy and Rando, 2005). Also, it has been identified as one of the risk factors for osteoporosis. Bone turnover is a coupled event of osteoblasts and osteoclasts, such that when one increases or decreases the other usually follows (Harada and Rodan, 2003). With age the balance is disrupted and bone lose becomes a major problem for the elderly population (Duque, 2008). It is not clear whether the ability to form bone tissue is lost because there is less

osteoblast cells in the marrow tissue or do they merely lose their ability to function, that is, to build bone. Some studies suggest that there is a small number of osteoblast cells but that they succeed in preserving their function, still the result is less bone formation (Stenderup, Justesen *et al.*, 2003). Other studies show that loss of bone comes from impaired cellular activity in bone signaling, showing decrease of autocrine and paracrine factors such as IGF-I IL-6 and TGF-B (Tanaka, Barnes *et al.*, 1996). In a different study it was noted that over time in the MSCs DNA that has been damaged and not repaired causes over time adult stem cell exhaustion that translates to loss of marrow cellularity and erthopoiesis (Nijnik, Woodbine *et al.*, 2007). Preclinical studies testing materials for use in bone tend to be performed in young adults animals, yet many of the clinical applications for those materials are for older patients. Therefore, there is a great need for materials that will promote faster healing in the aged group, as if they were young adults.

Porcine Bone Marrow Matrix (PBMM)

Porcine bone marrow matrix (PBMM) is a material produced by decellularizing porcine bone marrow matrix. Boston Scientific provided it to us in a sterile liquid suspension. We hypothesized that PBMM would provide a growth factor enriched scaffold that would enhance repopulation of the marrow cavity with multipotent stem cells. This was based on our studies using fetal porcine tooth germ derived enamel matrix derivative (Schwartz et al., 2000), which is also a decellularized matrix material that has been shown to promote periodontal regeneration in animals and humans (Venezia et al., 2004; Boyan et al., 2000). Moreover we hypothesized that the quality of the marrow produced in the presence of PBMM, in aged animals, would more closely resemble that seen in young animals. Because PBMM is a biomaterial, it was necessary to define the kinetics of

marrow restoration in immunocompromised rats. To test our hypothesis that PBMM would result in "young" marrow it was necessary to determine if kinetics of bone marrow restoration varied as a function of age.

Specific Aims and Experimental Design

The **overall goal** of this thesis was to investigate whether the primary bone formation of bone and its remodeling in the bone marrow ablation model is age dependent, and to establish a new way for measuring bone formation and resorption using μCT analysis. Also, to study the effect of a novel biomaterial on the marrow restoration in aged rats. The **general hypothesis** was that bone formation and its remodeling following bone tibia marrow ablation will be slower in aged animals and that porcine bone marrow matrix (PBMM) will enhance the restoration of the bone marrow tissue.

Aim 1: To study whether the induction of bone and its remodeling in the bone marrow ablation model is age dependent. The **objective** of this study was to examine the effects of age on marrow restoration and to compare μCT and histological methods. Our **hypothesis** was that bone formation and its remodeling following bone tibia marrow ablation will be slower in aged animals. To address this question we have used the bone marrow ablation model on three different age groups; young, adults and aged (one, three and ten) months rats. We then measured both primary bone formation and resorption using both methods; μCT and histological analysis.

Aim 2: To study the effect of PBMM on bone marrow restoration following ablation in aged animals. The **objective** of this study was to assess the effect of PMBB on marrow

restoration in aged rats. Ten-month-old nude rats were tested. The hypothesis for our study was that PBMM would enhance bone marrow regeneration. The rats had to be immunocompromised since a xenograft biomaterial was inserted to the tibia and we wanted to avoid the rejection of this material. A pilot study was done first in order to choose one time point that would then be used to test the material in a dose dependent manner.

CHAPTER 2

REGENERATION OF BONE MARROW AFTER TIBIAL ABLATION IS AGE DEPENDENT

Introduction

Injuries to the bone marrow initiate endosteal bone formation. This process is characterized by primary bone formation, resorption and marrow restoration (Boyan, 1993). The bone building cells that conduct primary bone formation are the osteoblasts. These cells derive from mesenchymal cells that are located at both the periosteum and the endosteum. They lay down osteoid, which is the organic matter of the matrix that eventually becomes calcified (Schwartz, 1995). The osteoclasts, bone resorbing cells, are then recruited through osteoblast secretion of growth factors to resorb the bone, leading to marrow tissue restoration. Bone turnover is a coupled event of osteoblasts and osteoclasts. With age, the balance is disrupted and bone lose becomes a major problem for the elderly population (Duque, 2008). However, it is not clear whether endosteal bone formation is affected by age although it is known that there are differences between the quality of the marrow within different age groups such as young adults and aged ones.

The bone marrow is a connective tissue consisting of two components: the hematopoieta and the stroma. The first gives rise to red blood cells and precursors of white blood cells. The second is composed of a mixture of multipotent cells from the mesodermal tissue. These cells differentiate to diverse cell linages such as osteoblasts, chondrocytes and adipocytes (Black and Woodbury, 2001). There are two types of marrow tissue; red marrow which consists of a majority of hematopoietic cells and

yellow marrow, which consists of mainly fat cells (Blebea, Houseni *et al.*, 2007; Fan, Hernandez-Pampaloni *et al.*, 2007). The ratio between the two different marrows changes with age in a normal physiological progressive conversion of red to yellow marrow (Ricci, Cova *et al.*, 1990).

The purpose of this research was to study the effect of age on bone marrow restoration and to propose a new model that will be based upon both μCT and histological analysis. In our study we used a bone marrow ablation model that mimics the healing process post injury to the marrow tissue. According to this model a part of the marrow in the tibial cavity is removed and endosteal bone formation is initiated (Bab, 1995). The model consists of four steps: at first blood clots to fill the marrow cavity; primary bone then replaces the blood; next remodeling of the primary bone by osteoclasts and then the marrow is then restored. Formerly, bone marrow ablation has been used in various ways to examine calcification during osteogenesis (Schwartz, Sela *et al.*, 1989; Marshall, Schwartz *et al.*, 1991), to study the effect of metal implants on osteointegration (Schwartz, Amir *et al.*, 1991; Schwartz, Swain *et al.*, 1992; Schwartz, Braun *et al.*, 1993; Sela, Gross *et al.*, 2000), and to investigate the effect of bone graft substitutes during marrow cavity regeneration (Schwartz *et al.*, 2008).

Age is known to be an important physiological factor that affects the healing process (Bak and Andreassen, 1989; Schwartz and Boyan, 1994; Fan, Crawford *et al.*, 2008). Most clinical studies to date test adults, but the healing process among different age groups is clearly not the same. We therefore decided to test three age groups: young, adult and aged. By adjusting this model for the employment of μCT in addition to traditional histological methods we gained the ability to compare these age groups. Thus

we were able to look at both bone formation and remodeling and as a result of that at the marrow restoration in an age dependent manner.

Understanding the progression in the elderly can give us an insight regarding the biological process of bone marrow restoration. New knowledge concerning the process could help in the development of new materials, which may be more suitable for the aged group, hopefully to be tested in clinical trials in the future.

Methods

Surgical Procedure

Total of 154 athymic male rats were used for the experiments. 56 ten-month-old retired breeder athymic rats were grouped as aged rats. 49 three-month-old rats were grouped as adult rats and 49 one-month-old rats were grouped as young rats. The Institutional Animal Care and Use Committee (IACUC) of the Georgia Institute of Technology approved the surgical protocol. Rats were anesthetized using 5% isoflurane inhalation. Once they were asleep, sleep was maintained with 2-2.5% of isoflurane. An intra-patellar incision was made on the left limb and a sub-periosteal flap was raised to expose the proximal aspect of the tibia. Using a #4 water-cooled dental burr at 20,000 RPM, a cavity was made in the proximal aspect of the tibia to allow access to the marrow cavity. Marrow was evacuated by repeated irrigation with saline solution. Bleeding was controlled using sterile gauze pads as needed. For our preliminary study of PBMM one group of the aged rats was injected with 0.2cc of PBMM in the marrow cavity. Wax was applied to this group only in order to keep the PBMM in the marrow cavity. Following the ablation of the marrow the periosteum and skin were closed using absorbable sutures

and wound clips. Rats were immediately given a pain killer shot and were place in a new cage. The right limb served as a control limb and was not operated on.

μCT Analysis

We evaluated the regeneration of the bone marrow tissue using μCT analysis. μCT is a method in which slides are reconstructed from a large number of projections at different angles using convolution projection algorithm, resulting in a high-resolution 3D image. It uses a focused X-ray beam, which passes through the sample and a computer creates an image depending on differences in sample density. Bone is very dense and absorbs a great deal of the X-rays. Measurements were done on a specific area of the tibial marrow cavity. From the middle of the whole tibia we took 50 slides up the metaphysis and 200 slides down the diaphysis. This is the area of the defect site where the new tissue was formed, and in which we were interested. The microarchitecture at the trabecular sites was determined. Parameters derived at the metaphysis and intramedullary canal included: tissue volume (mm3), bone volume (mm3), bone volume to tissue volume (BV/TV: %), trabecular number (#mm), thickness (mm) and spacing (mm). Harvest of the rats was done at 7 different time points: 0, 7, 14, 21, 28, 35 and 42 days. Both tibias were harvested. The left was the ablated one and the right was used as a control. Samples were placed in tubes with 70% ethanol for 24 hours. Then the samples were placed in formalin for at least 48 hours in room temperature. The samples were then scanned while in formalin for future histological analysis.

Histomorphometric Analysis

After the tibias were scanned they were placed back in formalin. The treated tibias were then prepared for histological analysis. The samples were first decalcified and then were embedded in paraffin. Two mid-sagital sections were made per tissue. The sections were stained with haematoxylin and Eosin. The first is a basic dye that has an affinity for the nuclei in the cell. Eosin is an acidic dye that will dye the cytoplasmic particles in the tissue including protein and extracellular matrix. For the histomorphometric measurements we used the computer program Image pro plus version 4.5.1. The area of interest was calculated from μCT measurements. Since every slice of the μCT was 21 micrometers we could calculate the length from the top tibia to where the defect area was. For example if the defect was around 300 slices- on histology it was: 21*300=6300 micrometer= 6.3 mm. This length was marked on the histological slide. Using the microscope 5X images were taken. Morphometric measurements were done on these areas. We measured the areas of the bone marrow, trabecular and cortical volumes and compared them to the total volumes of the frame. We took 6 samples per time point.

We were also interested in the fat content in the marrow cavity. Fat cells were seen as small blank circles. These were counted using the computer program by count objects tool.

Statistical Analysis

Statistical analysis of the results was made using analysis of variance (ANOVA). Differences between groups were determined using Bonferroni's correction to Students's t-test.

Results

The results of this study prove that marrow restoration is age dependent. This is truly important since many clinical trials generally use young and adults populations even though there is a significant difference in healing when it comes to aged population. In the future this should be taken into consideration when materials are being manufactured and marketed by the industry. There is a need for new materials that will specifically aim at the aged group.

μCT Analysis

μCT showed a lower amount of trabecular bone for the one month old rats compared to three and ten months old on day 0 controls (Figure 1). At day 7 there was a peak of trabecular bone formation in the one and three months old rats. However, the 10 month old rats exhibited peak bone formation on day 14. This suggests that bone formation and remodeling occurred more quickly in the younger animals.

This was confirmed by quantitative analysis. There was significant primary bone formation in the one-month-old rats on day 7 (Figure 2a). No differences were observed in the contralateral limb between days 0 to day 7 (Figure 2b). Comparison of treatment to control limbs again showed that peak bone formation was on day 7 and remodeling of the bone occurred by day 21 (Fig 2c).

Bone formation in the three months old rats also peaked on day 7, but remodeling occured between day 14 and day 35 (Fig 2d). No changes in the contralateral limb were

observed (Fig 2e), resulting in treatment/control ratios comparable to the treatment limb (Fig 2f).

Ten-month-old rats had a delay in bone formation when compared to adults and young rats. Peak bone formation was at day 14 (Fig 2g). There was no change in the contralateral limbs (Fig 2h). However, when we compared treatment limbs to the control limbs, bone formation was increased on days 7 and 14 and remodeling occured on day 28 (Fig 2i).

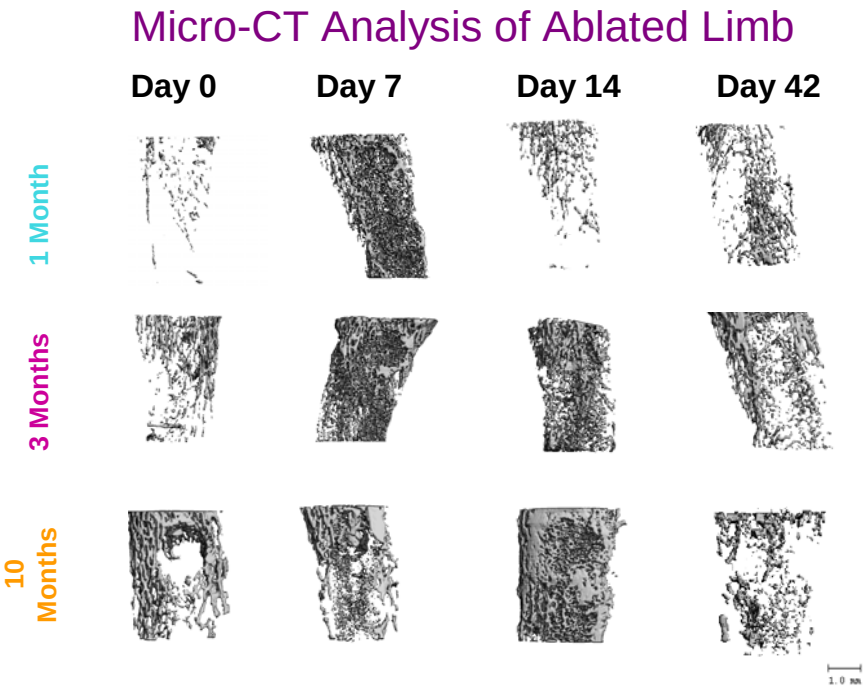

Figure 1: µCT images of trabecular bone in treated limb

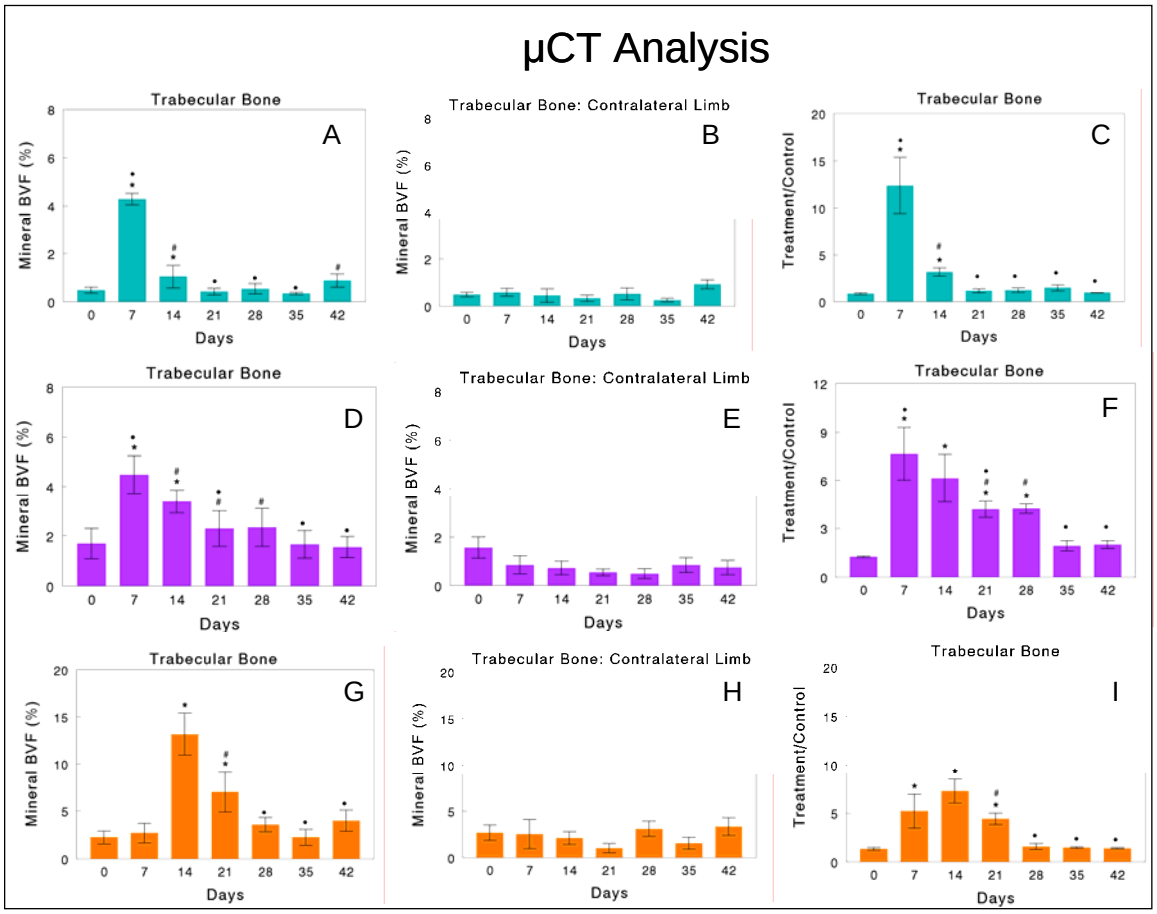

Figure 2: Effect of age on trabecular BV/TV of the rat tibial marrow canal; increase in bone formation on days 7, 14 and 42 in 1 month old (2A), Control limb (2B), increase in bone formation on days 7 and 14 only when compared to contralateral tibia in 1 month old (2C), Increase of bone formation on day 7 up to day 28 in 3 month old (2D), Control limb (2E), increase in bone formation on day 7 up to day 28 compare to contralateral tibia in 3 month old (2F), increase bone formation starts on day 14 and lasts until day 21 only in 10 month old (2G) Control limb (2H), increase in bone formation from day 7 to day 21 when compare to contralateral tibia in 10 month old (1I). Data are means + SEM

Histomorphometric Analysis

Figure 3 is a representative micrograph demonstrating the presence of primary bone. It can be seen that the older animals have more fat cells compared to young ones. Histomophometric analysis demonstrated peak bone formation on day 7 in the young animals (Fig 4a), and complete restoration of marrow on day 14 (Fig 4b). For the adult group the peak of bone formation also occured on day 7, but on day 14 bone formation was further increased (Fig 4c). The marrow was restored by day 21 (Fig 4d). In the aged group there was increased bone formation on both days 7 and 14 (Fig 4e). The aged rats restored their marrow on day 28 (Fig 4f).

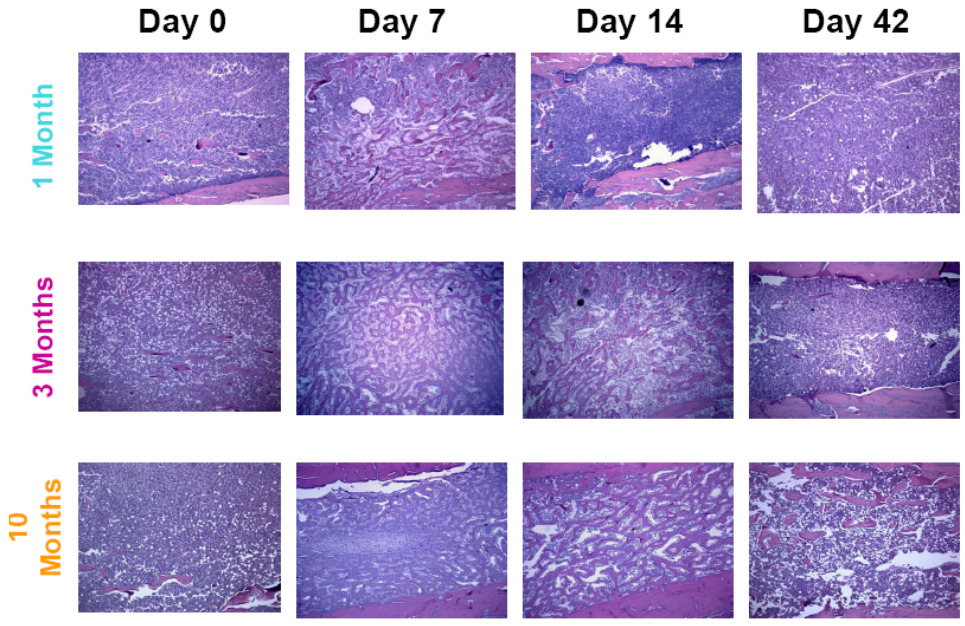

Figure 3: Histological images of trabecular bone in treated limb stained with H&E

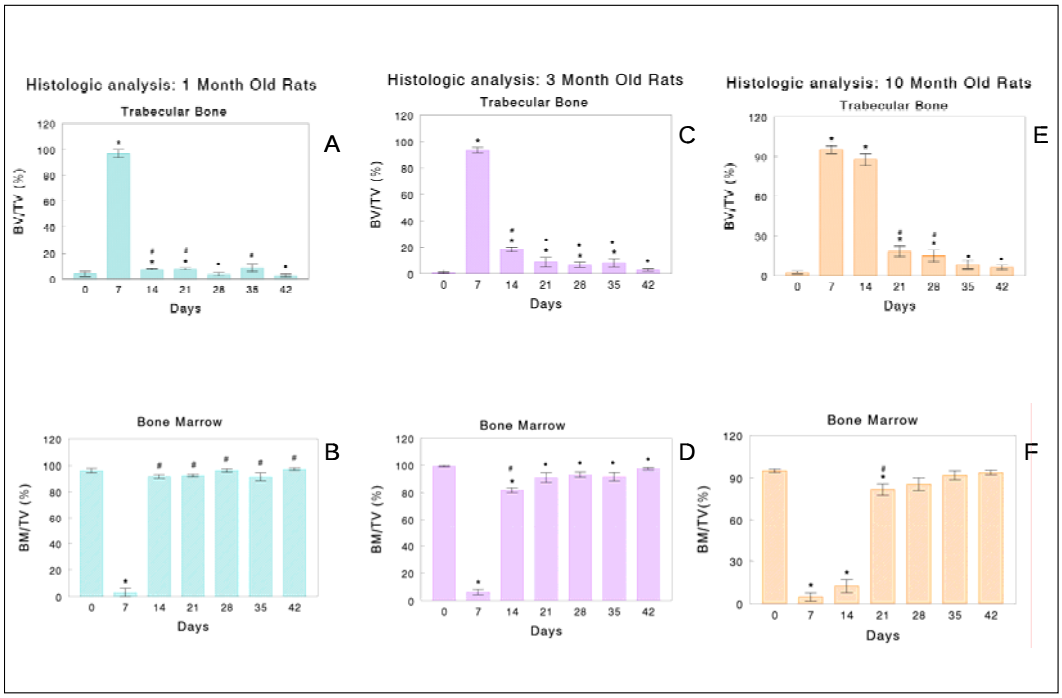

Figure 4: Effect of age on trabecular restoration (BV/TV) and marrow restoration (BM/TV) of the rat tibia; increase of trabecular formation on day 7(4A), deceases of marrow tissue on day 7 in 1 month old (4B), Increase of trabecular formation on day 7 and day 14 in 3 months old (4C), decrease in marrow tissue on day 7 and day 14 in 3 month old (4D), increase in trabecular on day 7, 14, 21 and 28 in 10 months old (4E) decrease in marrow tissue on day 7, 14 and 21 in 10 month old (4F). Data are means + SEM N=6

Age Dependent Changes in Fat Cell Number

The fat content per marrow cavity increased as the animals aged. For the young group only a small amount of cells appeared to be fat cells as part of the whole marrow cells diversity. On day 21, there was an increase in the fat cell numbers whereas on day 28 there was significant difference among the different age groups. Adults and aged rats appeared to have the same number of fat cells. This might suggest that the conversion to fat marrow occurs around three months old.

Table 1: Fat cell number within the marrow cavity. On day 0, 7 and 14 there is a lower amount of fat cells for 1 month group when compare to 3 and 10 months. On day 21 the number of fat cells is increasing for the 1 month old. On day 28 there is no significant difference between the 3 age groups. Data are means ± SEM N=6.

Number of Fat Cells Per Cavity

Day	1 Month	3 Months	10 Months
0	9 ± 4#	334 ± 115	366 ± 64
7	2 ± 1#	34 ± 18*	0*
14	38 ± 24*#	228 ± 30	181 ± 52*
21	168 ± 39*#	354 ± 79	312 ± 82
28	221 ± 60* [131 ± 35]	327 ± 55	173 ± 52*
35	485 ± 100*	407 ± 96	316 ± 89
42	378 ± 90* [197 ± 40]	440 ± 127	438 ± 94

[ ] = control limb

Cortical Bone

Remodeling occurs constantly throughout life. However, fast remodeling occurs when growth to form a mature bone is still ongoing. Bone marrow ablation might interfere with this process as the hole that is being drilled is on the axis at the time that remodeling is active, thus we would expect to see an effect of our treatment when we use young rats. There was an increase in the total volume of cortical bone in one-month-old rats. On day 42 the volume of the cortical bone was restored to its normal volume. However, in adult and aged rats there was a delayed increase of total volume and bone volume remained high (table 2). Interestingly, the young rats did not exhibit increased bone formation that remained elevated as was the case with total bone volume. In contrast, the new bone formation in adult and aged rats remained longer and the cortical bone volume also remained elevated (table 3).

Tables 2, 3; cortical analysis; total volume treated over contralateral limb (2) bone volume treated over contralateral (3)

Cortical Analysis

Treatment over Contralateral limb: Total Volume

Day	1 Month T/C	3 Month T/C	10 Month T/C
0	0.99 ± 0.02	1.01 ± 0.03	0.99 ± 0.01
7	1.48 ± 0.05 *	1.07 ± 0.01 *	1.02 ± 0.02
14	1.36 ± 0.03 *	1.18 ± 0.04 *	1.10 ± 0.03 *
21	1.32 ± 0.07 *#	1.19 ± 0.01 *	1.19 ± 0.03 *
28	1.18 ± 0.02 *#	1.20 ± 0.02 *	1.21 ± 0.02 *
35	1.09 ± 0.05 #	1.17 ± 0.02 *	1.24 ± 0.04 *
42	1.00 ± 0.02 #	1.20 ± 0.02 *	1.18 ± 0.02 *

Treated leg over Contralateral: Bone Volume only

Day	1 Month T/C	3 Month T/C	10 Month T/C
0	0.974 ± 0.014	0.938 ± 0.017	0.958 ± 0.021
7	1.039 ± 0.026	1.032 ± 0.010	0.970 ± 0.014
14	1.100 ± 0.013 *	1.126 ± 0.026 *	1.064 ± 0.026
21	1.160 ± 0.186	1.148 ± 0.018 *	1.148 ± 0.033 *
28	1.031 ± 0.014	1.136 ± 0.012 *	1.175 ± 0.026 *
35	0.998 ± 0.029	1.154 ± 0.017 *	1.195 ± 0.034 *
42	0.959 ± 0.005	1.168 ± 0.017 *	1.157 ± 0.017 *

Discussion

Endosteal bone formation and remodeling are age-dependent. Aged rats have a delayed response in new bone formation and resorption. Young rats respond faster in both formation and resorption. Decline in tissue repair could arise from age related changes in the environment or in the niche of the stem cells, for example an increase in fat cells (Rando, 2006). Therefore, there is a clinical need for new materials that can induce healing in aged population. It is well documented that this process in older population takes longer and is often not fully successful. In this study we compared the capability of rats to restore marrow after marrow removal. We looked at two processes: primary bone formation, and the remodeling of the bone resulting in marrow restoration. In this study we used μCT for the first time to evaluate the calcified bone after removal of marrow. We were concerned about the limitation of this method as for its ability to detect primary bone formation since it may not be as calcified as trabecular bone. We found that μCT is highly sensitive to small changes in tissue calcification; therefore we were able to follow trabecular bone restoration as a function of time and age. We scanned both the treated limb and the untreated limb to make sure that an ablation effect and not natural limb growth, was investigated. The control limb was also used to eliminate an effect of position, since the hole where the marrow was aspirated was positioned at slightly different locations from animal to animal. The μCT provided us with important information that has not been formerly noticed using histological sections alone. The μCT also gave us the total volume of the tibia, which helped to determine the complete area of the calcified tissue in the marrow cavity. Choosing a threshold that will represent all age groups was necessary. This was not an easy task since tissue calcification varies

with age. For that reason we looked at several thresholds, finding that there is no difference in the course of bone formation. Thus we were assured that no bone was lost or that no extra bone was counted thus avoiding background artifact. Our histology analysis corresponded with our μCT data. However, we were able to determine the quality of the marrow itself. Using H&E stain we were able to detect trabecular as well as bone marrow cells such as red cells and fat cells. We were unable, however, to distinguish between the calcified and non-calcified tissue, consequently we can only point to the formation of a new bone. The formation of the bone and its remodeling was delayed in aged rats but with the control limb, focusing on fat cells, a significant difference between adult and aged groups was not detected. This might suggest that there are several additional factors that determine the restoration of marrow not only fat. One should perhaps investigate more in depth marrow quality after being restored in terms of its full potential to function as new bone marrow. It would also be interesting to look at the mesenchymal stem cells (MSC) specifically to see whether they express MSC characteristics. The composition of the marrow may also change with age and it may be important to see if new marrow regains its younger look or will it advance to a more mature phenotype. Various studies are currently trying to identify whether the numbers of MSC's are decreasing with age or do they merely lose their ability to dedifferentiate to bone building cells. Interestingly, the cortical bone analysis gave us new information. Whereas for the young rats the total volume of cortical was significantly increase at the early time points of our study the total volume was reduce to its normal volume. When we then looked the bone formation only, their bone formation was not as high as we expected it would be, which means that the young animals compensate in their total

volume for their lost of marrow. However, for the adult and aged there might be a lower amount of total volume increase but this is a real increase in the bone formation and we believe this is the reason that the cortical formation stays high throughout this process.

The significance of this study is in the development of a model that enables us to view bone marrow restoration in aged rats. Using this model we can examine various materials such as implant and bone grafts to learn more about bone recovery processes in general and also to test materials that might induce healing in older populations, in particular. As Science advances so does human life expectancy, the aged population around the world is growing, in order to guaranty quality of life one will need to fully understand bone healing in elderly population.

CHAPTER 3

THE EFFECT OF PORCINE BONE MARROW MATRIX ON MARROW RESTORATION

Introduction

There is a great clinical need for materials that can either augment or substitute for autologous bone graft. A goal for those materials is that they be osteogenic, causing bone to form across defects that are poorly apposed. Many of the currently available bone substitutes, including allografts, xerographs and alloplasts, meet this goal, providing adequate osteoconductive surfaces to support migration of osteoblast progenitor cells and their differentiation into bone forming secretory osteoblasts.

With greater understanding of bone biology and bone repair, it has become desirable to identify materials that resorb as new bone is formed, thereby facilitating the restoration of normal bone contours and biomechanics. Most bone graft materials that are used in dental implantology and oral and maxillofacial surgery, as well as orthopaedics, are placed in sites that normally remodel once bony union has been achieved, resulting in restoration of the marrow cavity and regeneration of marrow. The resorption of primary bone within the marrow cavity can occur more rapidly than the remodeling of primary bone into lamellar bone in the cortex, and recent studies show that bone graft materials can impact this both positively and negatively (Doukarsky et al., 2008). Thus, it is of importance to develop materials that can promote marrow restoration at an appropriate rate, resulting in normal healthy marrow.

Preclinical studies testing materials for the use in bone tend to be performed in young adults animals, yet many of the clinical applications for these materials are in older patients. The quality of bone marrow changes with age, becoming fattier and the type of marrow fat that is present in older individuals tends to be “yellow”. Moreover, there is a reduction in the number of mesenchymal stem cells present in the marrow stroma. It is not known if there are age dependent differences in the rate of marrow restoration or in the quality of the marrow that forms. It also is not known if materials used to enhance bone healing alter the quality of marrow.

To address these questions, we took advantage of the rat tibial marrow ablation model developed by us previously to study endosteal bone formation and remodeling adjacent to biomedical implant materials (Schwartz, Amir *et al.*, 1991; Boyan, Schwartz *et al.*, 1993; Schwartz, Braun *et al.*, 1993), and by others to assess the effects of bone anabolic and catabolic agents (Suva, Seedor *et al.*, 1993). In this model, the tibial marrow is removed and a blood clot and granulation tissue from within the marrow cavity over the first three days of healing. In 300g Sprague Dawley rats, by day 6, primary bone forms on the endosteal surface and eventually fills the marrow cavity. Beginning at day 12 and continuing through day 25, remodeling occurs, resulting in resorption of the primary bone. By day 35, replacement of the primary bone with bone marrow is complete, resulting in regeneration of the normal marrow tissue.

Porcine bone marrow matrix (PBMM) is a material produced by decellularizing porcine bone marrow. Boston Scientific provided it to us in a sterile liquid suspension. We hypothesized that PBMM would provide a growth factor enriched scaffold that would enhance repopulation of the marrow cavity with multipotent stem cells. This was based

on our studies using fetal porcine tooth germ derived enamel matrix derivative (Schwartz, Carnes *et al.*, 2000), which is also a decellularized matrix material that has been shown to promote periodontal regeneration in animals and humans (Boyan, Weesner *et al.*, 2000; Venezia, Goldstein *et al.*, 2004). Moreover we hypothesized that the quality of the marrow produced in the presence of PBMM, in aged animals, would more closely resemble that seen in young animals. Because PBMM is a biomaterial, it was necessary to define the kinetics of marrow restoration in immunocompromised rats. To test our hypothesis that PBMM would result in "young" marrow it was necessary to determine if kinetics of bone marrow restoration varied as a function of age.

Methods

This study was conducted in two parts: characterization of aged rat model and assessment of PBMM effectiveness. The first part was conducted as a preliminary study where aged rats were studied and PBMM was injected to one group and was also characterizes. From the results of this study we chose one time point for the main experiment where the effective of PBMM was tested. In both parts the marrow restoration was characterized using both μCT and histological slides methods. The surgery was conducted in the same way in both experiments. A total of 99 ten-month-old retired breeders nude rats were used.

Animal Model

Male nude rats one, three and 10 months of age were purchased from Harlan. Marrow was ablated in the right hind tibia and at 0, 7, 14, 21, 28, 35 and 42 days, rats were euthanized, and treatment and control tibias harvested. In addition, we assessed the

handling properties of PBMM and the biological response to PBMM in a preliminary study using a high dose of PBMM. This group of 7 aged rats was euthanized at 42 days.

Test Article

For the pilot study, PBMM was supplied to us at a concentration of 31.3 mg PBMM/ml PBS. This was diluted to a concentration of 20 mg/ml and each rat received a dose of 0.2 ml/marrow cavity. For the dose-response study, PBMM was provided at a concentration of 20 mg/ml was used in its undiluted form or was diluted 1:10 v/v with PBS. Once a time point was chosen for the dose-response study, a total of 5 groups were used for the experiments. The first was control ablation on day 0. The second was the control of the specific day of the study- an empty group, the third was a vehicle that contained the PBS only. In the forth a low dose of PBMM 2mg/ml was used and in the fifth a high dose of PBMM 20mg/ml per marrow cavity was used.

Surgical Procedure

The surgical protocol was approved by the Institutional Animal Care and Use Committee at the Georgia Institute of Technology. This protocol has been described in detail in previous studies (Schwartz, Amir *et al.*, 1991; Boyan, Schwartz *et al.*, 1993; Schwartz, Braun *et al.*, 1993). Rats were anesthetized using isoflurane. An intra-patellar incision was made and a sub-periosteal flap was raised to expose the proximal aspect of the tibia. Using a #4 water-cooled dental burr at 20,000 RPM, a cavity was made in the proximal aspect of the tibia to allow access to the marrow cavity. Marrow was evacuated by repeated irrigation with saline solution. PBMM was inserted by injection into the marrow cavity eight of the rats (0.2 ml/marrow cavity). Bone wax was used to seal the bone in the PBMM treated animals, both in the pilot study and in the dose-response

study. In addition, in the dose-response study, all control animals also received a bone wax seal at the drill site. Bleeding was controlled using sterile gauze pads as needed. At the end of the surgical procedure, the periosteum was closed using 4/0 absorbable sutures and the skin was closed using wound clips. Buprenorphine was given as a post-operative analgesic.

MicroCT

The extent of endosteal bone formation in the ablated limbs was determined by μCT. Immediately after harvest, the bones were fixed in 70% ethanol for 24 hours and post-fixed in neutral buffered formalin. In order to obtain an accurate assessment of the amount of new trabecular bone and its resorption it proved necessary to use the contralateral limb as an internal control. The amount of marrow was assumed to be marrow space not filled with trabecular bone. The micro architecture morphology at the trabecular sites was determined. Parameters derived at the metaphysis and intramedullary canal included: tissue volume (mm3), bone volume (mm3), bone volume to tissue volume (BV/TV: %), trabecular number (#mm), thickness (mm) and spacing (mm). To determine if this assumption was valid, histomorphometric measurements were made as described below.

Histology

After the tibias were scanned they were placed back in formalin. The treated tibias were then prepared for histological analysis. Samples were decalicified and embedded in paraffin. Two mid-sagittal sections were made per tissue. The sections were stained with haematoxylin and eosin. For the histomorphometric measurements we used the computer program Image pro plus version 4.5.1. Fat cell number per marrow cavity was also

measured using the Image pro plus version 4.5.1. We first converted the image to black and white. Then using the future *count object* we were able to choose the fat cells only using their round feature.

Results

Pilot Study

Micro-CT analysis showed that PBMM had an effect on marrow restoration (Figures 5a, 5b). However, there was no statistical difference between the aged groups (control) on day 42 and the ones treated with PBMM of the same day (42). But when we examined the effect of PBMM using histological analysis, it looked that the PBMM did have a positive effect and indeed the amount of trabecular bone was lower than the control of day 42 (Figure 4c, 4d). Bone marrow levels were also significantly higher in the PBMM group. Based on these results we chose an earlier time point to investigate whether PBMM can hasten bone marrow regeneration. We chose day 28 as our goal, since aged rats seemed to restore their marrow already on day 35.

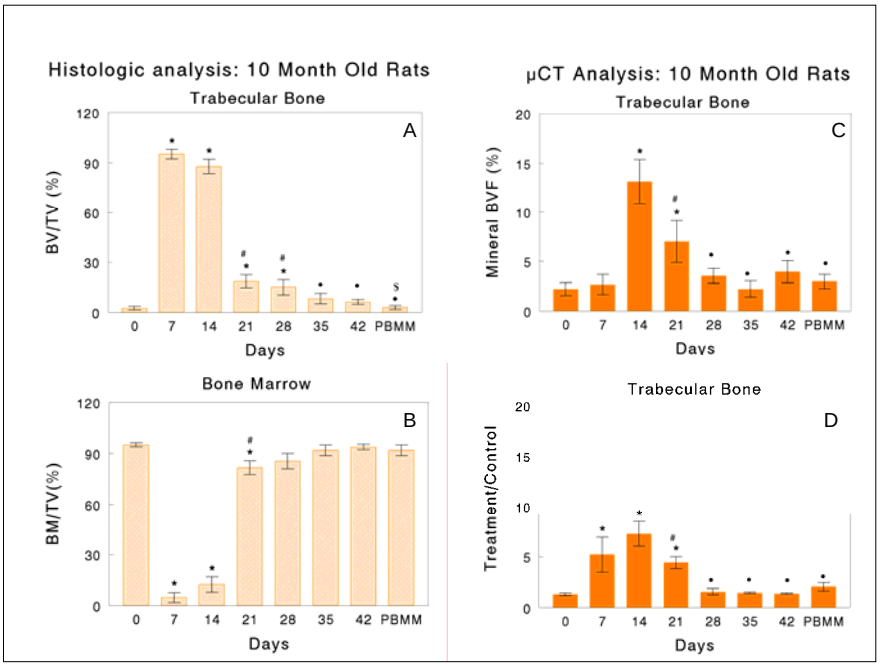

Figure 5: analysis of the effect of PBMM; histological section show a lower amount of trabecular bone formation on day 42 with PBMM compate to control (5A) bome marrow restoration (5B) CT analysis show no difference in trabecular boneof the effect of PBMM (5C, 5D)

PBMM Dose Study

In addition, we took measurements of bone formation and marrow restoration both above and below the bone wax seal (Figure 6). This was necessary since the wax interfered with bone healing across the defect site. The results of these analyses showed us that PBMM had no effect on marrow restoration at that time, either in the diaphysis or in the metaphysic (Figure 7). The µCT data were confirmed by histo-morphometry measurements. Moreover, there was no change in fat cell number that could be attributed to the presence of the PBMM.

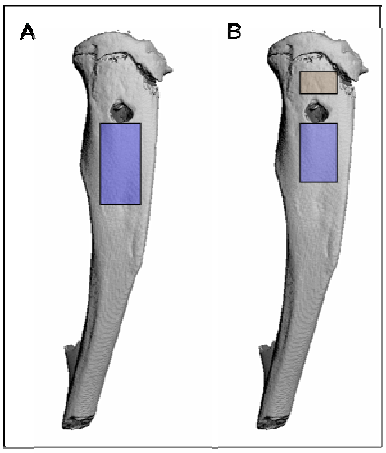

Figure 6: Different way to assess the bone marrow: purple old measurements, brown new measurements.

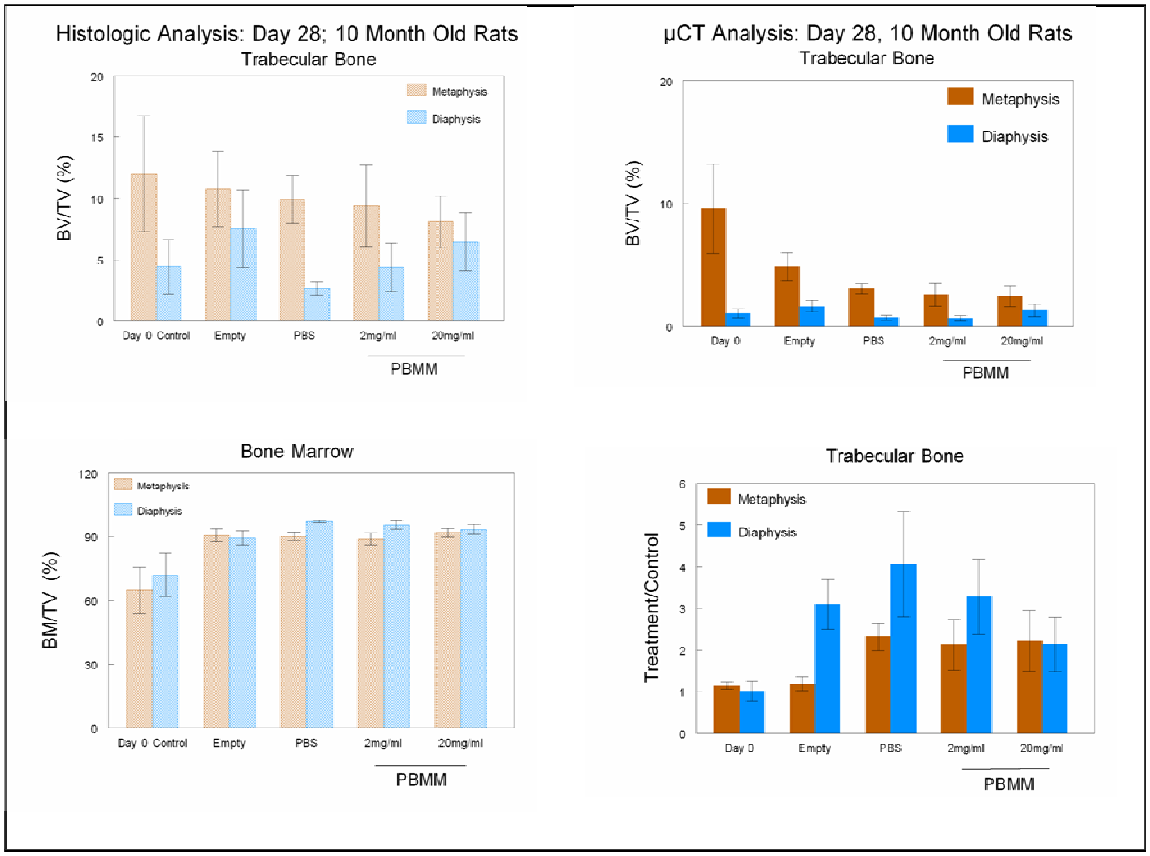

Figure 7: PBMM dose dependent study show no increase in bone marrow compare to empty both using CT and histological analysis

Discussion

In the first part of this study we looked at aged rat's marrow restoration as compared to young and adult ones. The results obtained showed, for the first time, the existence of differences in the kinetics of endosteal bone formation and remodeling, in the tibial marrow cavity of nude rats. μCT analysis indicated that very young rats exhibit rapid bone formation that occurs primarily in the treatment limb, followed by rapid bone resorption and restoration of the marrow cavity. Adult rats exhibited rapid bone formation, but remodeling of that bone occurs at a slower rate than in young growing rats and the systemic effect of marrow ablation is greater. Aged rats exhibit delayed bone formation coupled with rapid bone remodeling.

The μCT and histological results indicate that PBMM does not hinder restoration of the marrow within the medullary canal in aged nude rats and that the normal endosteal contours are restored as well. However, the results also show that PBMM does not appear to promote restoration of marrow as originally hypothesized.

The preliminary study suggested that PBMM caused more rapid restoration of marrow than was seen in the untreated animals at 42 days. In the PBMM treated animals, the amount of bone remaining in the marrow cavity at 42 days was less than in the untreated animals and was comparable to that seen at time 0. Histology permitted a clearer view of the tissue at the interface of bone and marrow than we were able to achieve with μCT, even after using different threshold values, which may have contributed to differences in the two data sets. In addition, we were better able to discriminate between pre-existing trabecular bone and new trabecular bone produced as a consequence of the ablation. By comparing the bone volume/total volume in the treated

limb to that of the contralateral control limb, we removed some of the potential error in the μCT data, but the final assessment afforded by this approach was not as precise as could be attained by histology. It is not known whether the result measured at 42 days reflected a change in bone resorption that occurred after day 28. The data from the earlier time point clearly failed to show a beneficial effect of the material.

At this point in the analysis, we cannot yet state whether aspects of the bone marrow other than amount or number of fat cells are affected by PBMM.

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