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Abstract

Acid bone lysates represent the growth factors and other molecules released during autologous graft resorption. However, the impact of these bone-derived growth factors on the healing of bone defects has not yet been investigated. The aim of the present study was, therefore, to examine the impact of acid bone lysates adsorbed to collagen membranes on bone regeneration. To this end, in 16 female Sprague Dawley rats a standardized 5-mm-diameter critical size defect on the calvarial bone was created. The defects were covered with collagen membranes that had been soaked either in serum-free media or acid bone lysates followed by lyophilization. After a healing period of four weeks, micro computed tomography (CT) and histological analyses by means of undecalcified thin ground sections were performed. Micro CT analysis of the inner 4 mm of the calvaria defect showed a greater bone defect coverage in the control group when compared to acid bone lysate group, 29.8 % (CI: 17.7 - 50.3) versus 5.6 % (CI: 1.0 - 29.8, p=0.03), respectively. Moreover, we found significantly more absolute bone volume in the control group when compared to acid bone lysate group, 0.59 mm^3 (CI: 0.27 - 1.25) versus 0.07 mm^3 (CI: 0.06 - 0.59, p=0.04), respectively. Histomorphometry confirmed these findings with a relative bone volume in the central compartment of 14.1% (CI: 8.4 - 20.6) versus 5.6 % (CI: 3.4 - 7.9, p=0.004) respectively. These findings indicate that bone-derived growth factors contained in acid bone lysates are able to attenuate bone regeneration within collagen membranes.

Keyword: bone regeneration, growth factors, bone grafts, bone allograft

Introduction

Bone augmentation have become a standard procedure driven by the need to increase bone volume in atrophic jaws prior to implant placement. One common procedure for bone augmentation is the use of autologous bone grafts, either as bone blocks or as bone chips.[1] Autologous bone grafts however, undergo resorption leading to the shrinkage of the bone block or the disappearance of the bone chips.[1] Up to one-quarter of the original graft size can be resorbed at the time of implant placement.[2] This osteoclastic resorption occurs during the early graft consolidation process at the augmented site.[3] Despite its susceptibility for catabolic changes, autologous bone is still considered the gold standard for bone augmentation, mainly due to the enhanced performance compared to bone substitutes during the early phases of bone healing.[4] Apart from the favorable osteoconductive properties of autografts, it is widely believed that the growth factors released by osteoclasts during graft resorption, including TGF-β, support the process of bone regeneration.

TGF-β is one of the most abundant cytokines in the bone matrix[5] which can be released during bone resorption and by acidic conditions.[6-8] TGF-β is then capable of binding to dental implants and collagen membranes.[9,10] Nevertheless, the role of TGF-β is not so clear as it can support but also hinder bone regeneration. For example, bone-derived TGF-β recruits mesenchymal cells, the progenitors of bone-forming osteoblasts, to the site of bone remodeling.[11] Similarly, TGF-β initiates and promotes heterotopic ossification in mice via recruiting mesenchymal progenitors.[12] TGF-β1 induces bone closure of rabbit skull defects[13] and TGF-β1-loaded implants provoke an increased bone surface area in a rabbit cranial defect model.[14] In contrast, TGF-β1 loaded β-TCP failed to support bone formation in a rat calvaria model[15] and in vitro, TGF-β reduces osteogenic differentiation in cell culture models.[16] High-doses of TGF-β1 also dampened bone regeneration by repressing the bone morphogenetic protein 2 (BMP2) activity.[17] Considering that acid bone lysates are a rich source of TGF-β that binds to collagen[6] the question arises whether the local application of acid bone lysates supports or hinders bone regeneration.

We have recently characterized the molecular composition of acid bone lysates prepared from porcine bone chips,[6] and showed that activation of the TGF-β signaling pathway is the major response of mesenchymal cells upon their exposure to acid bone lysates, also causing the expected decrease of in vitro osteogenic differentiation.[6] Since TGF-β adsorbs to collagen[18] and thus to the classical collagen barrier membranes,[10] these membranes can be considered as suitable carriers to investigate acid bone lysates on bone regeneration in vivo. Using this approach, we have recently shown that bone-conditioned medium lyophilized onto collagen membranes slightly reduces bone formation in rat calvaria defects.[19] Taking advantage of this established model, the aim of the present study is, therefore, to examine the impact of acid bone lysate adsorbed to collagen membranes on bone regeneration in rat calvaria defects.

Material and Methods

Study design

The present study was conducted at the Department of Biomedical Research of the Medical University of Vienna following the ARRIVE guidelines [20]. Before starting the study, an approval of the study protocol was obtained by the local ethical committee at the Medical University of Vienna (GZ BMWFW-66.009/0217-WF/V/3b/2015). Briefly, sixteen 7-month old (200-300g) female Sprague Dawley rats from the Division for Biomedical Research (Himberg, Austria) were randomly allocated into two groups with 8 animals each: control group received collagen membranes soaked in serum-free medium (SFM) and acid bone lysates group received collagen membranes soaked in acid bone lysates. Collagen membranes (25 mm x 25 mm; Bio-Gide®, Geistlich, Wolhusen, Switzerland) were loaded with SFM or with 1 ml of pooled acid bone lysates and frozen at -80°C. Lyophilization was then performed using a freeze dryer Alpha 1-2 LDplus (Martin Christ, Osterode am Harz, Germany). Randomization was performed via a computer-generated randomization. The animals were treated according to the guidelines for animal care with free access to water and a standard diet.[21]

Acid bone lysate

Acid bone lysate (ABL) was prepared as recently described.[6,9] Bone was obtained from adult pigs within 6 h post-mortem (Fleischerei Leopold Hödl, Vienna, Austria). Bone chips from the mandible, calvaria, and tibia were harvested with a bone scraper (Hu-Friedy, Rotterdam, the Netherlands). Thereafter, the bone chips were cleaned using Dulbecco's modified Eagle medium (DMEM) that was supplemented with antibiotics (Invitrogen Corporation, Carlsbad, CA, USA). Five gram of wet bone chips were incubated while being stirred with 50 ml of 0.1 N HCl (10% weight/volume) at room temperature. Acid bone lysates were harvested after 16 h, centrifuged, and then pH neutralized. Subsequently, another centrifugation was performed. Acid bone lysates were then filtered sterile using a 0.2 µm syringe filter (VWR International, Pennsylvania, USA) and kept frozen at −80 °C. Right before each experiment, the stocks were thawed.

Surgical procedures and postoperative treatment

The surgical procedures were performed as previously described.[19] Briefly, all rats received ketamine (50 mg/kg i.p.) (AniMedica, Senden, Erlangen, Germany) and xylazine hydrochloride (10mg/kg i.p) (Bayer Austria, Vienna, Austria). A standardized 5-mm-diameter critical size defect was created on the calvaria bone by the use of a trephine burr (Medos Medizintechnik; Vienna, Austria). The collagen membrane was trimmed and placed onto the defects. The membrane overlapped the walls of the defect by at least 1 mm. Thereafter, the membrane was stabilized and the flap was sutured in layers with resorbable sutures (Vicryl 5-0; Ethicon GmbH, Norderstedt, Germany). Buprenorphine 0.06 mg/kg, (Temgesic®, Temgesic, Reckitt and Colman Pharm., Hull, UK) and piritramide in drinking water ad lib was administered for pain relief. After four weeks of healing, animals were sacrificed by an intracardial overdose of sodium pentobarbital (300 mg/kg). Samples from each calvarium was obtained and further processed for micro computed tomographic (µCT) and histological analysis.

Micro CT analysis

After euthanasia the 16 heads were fixed in phosphate-buffered formalin (Roti-Histofix 4%, Carl Roth, Karlsruhe, Germany). Micro CT was carried out at 90 kV/200 µA with an isotropic resolution of 17.2 µm and an integration time of 500 ms (µCT 50 Scanco Medical AG, Bruttisellen, Switzerland). The images were rotated using Amira 6.2 (Thermo Fisher Scientific, Waltham, USA) to obtain the drill direction in the Z axis with the defect near the center of the image. Via the Definiens Developer XD2® software (Munich, Germany, Version 2.1.1), the region of interest (ROI) was segmented from the µCT images with an individually developed ruleset. The mineralized tissue within the inner 4mm of the defect was measured.

Histological and histomorphometric analysis

The 16 samples were dehydrated with ascending alcohol grades and embedded in light-curing resin (Technovit 7200 VLC + BPO; Kulzer & Co., Wehrheim, Germany). Blocks were further processed using EXAKT cutting and grinding equipment (Exakt Apparatebau, Norderstedt, Germany). Thin-ground sections from all samples were prepared in a plane parallel to the sagittal suture and through the center of the defect and stained with Levai–Laczko dye. The slices were scanned using an Olympus BX61VS digital virtual microscopy system (DotSlide 2.4, Olympus, Japan, Tokyo) with a 20x objective resulting in a resolution of 0.32 µm per pixel and then quantified using Adobe Photoshop® software (Adobe, San Jose, CA, USA). Histomorphometric analysis was performed at three ROIs representing (i) the central compartment within the defect margins, (ii) the adjacent ectocranial compartments, and (iii) the outer compartment on the surface of the host`s cortical bone.

Statistics

Statistical analysis was based on the data observed with the µCT and histomorphometric analysis. The Shapiro–Wilk test was used to test the normality of the data sets. For µCT, median values and confidence intervals (CI) of the primary outcome (% of bone defect coverage) and the bone volume (BV) between control and test group were compared with Mann-Whitney U test due to the distribution of the data. For histomorphometry, BV per tissue volume (BV/TV in %) between control and test group were compared with Mann-Whitney U test. Analyses were performed using Prism v7 (GraphPad, La Jolla, CA). Owing to the pilot nature of the study, the sample size was chosen based on experience from previous studies[19] to balance the ability to measure significant differences while reducing the number of animals used. Significance was set at $p<0.05$.

Results

µCT analysis

Figure 1 shows three representative samples per group of the calvaria defect, corresponding to the minimum (A,B), median (C,D) and maximum (E,F) value in terms of bone regeneration. The control group displayed higher amounts of bone volume compared to the acid bone lysate group. Moreover, there was a subjective impression of a bone formation pattern, possibly caused by the fibrils of the collagen membrane (**Figure 1**). Quantitative analysis of the inner 4 mm of the defect showed that the relative bone area was significantly higher in the control group than in the collagen membranes soaked and lyophilized with acid bone lysates, 29.8% (CI: 17.7 - 50.3) versus 5.6 % (CI: 1.0 - 29.8), respectively (P=0.03) (**Figure 2A**). Quantitative analysis further displayed that also the bone volume was significantly higher in the control group compared to the acid bone lysate group, 0.59 mm^3 (CI: 0.27 - 1.25) versus 0.07 mm^3 (CI: 0.06 - 0.59), respectively (P=0.04) (**Figure 2B**). Taken together, these findings suggest that acid bone lysates lyophilized onto collagen membranes reduce bone formation in a rat calvarial defect model.

Histomorphometric analysis

Figure 3 shows three representative ground sections per group of the calvaria defect, corresponding to the minimum (A,B), median (C,D) and maximum (E,F) value in terms of bone regeneration according to the µCT analysis. In agreement with the µCT analysis, the control group showed more bone formation as compared to acid bone lysate group. To confirm these subjective impressions, an histomorphometric analysis was conducted (Figure 4A). The histomorphometric analysis revealed that in the central compartment of the defect, the percentage of bone volume per tissue volume (BV/TV) was significantly higher in the control group compared to acid bone lysate group, 14.1% (CI: 8.4 - 20.6) versus 5.6 % (CI: 3.4 - 7.9, p=0.004), respectively (Figure 4B). Next, and in order to determine whether the effect of acid bone lysate was restricted to the central compartment, two other regions of interest were analyzed. The percentage of BV/TV did not differ between the control and acid bone lysate group, neither in the ectocranial compartments of the defect, 8.0% (CI: 4.6 - 14.9) versus 7.5% (CI: 1.9 - 10.5, p>0.05) (Figure 4C), nor in the outer compartment encompassing the external surface of the defect`s margin, 14.2% (CI: 9.0 - 20.4) versus 15.4% (CI: 8.1 - 29.13, p>0.05), respectively (Figure 4D). Overall, these observations indicate that the effects of acid bone lysates are restricted to the area encompassed by the collagen membrane.

Histological analysis

Histological analysis confirmed previous findings that bone formation mainly occurs inside the collagen membrane (**Figure 5**).[19] The fibers of the original collagen membrane (light pink) are either surrounded by the new bone or soft tissue. The calvarial defect in the control group was mainly filled by woven bone (dark purple). This woven bone formed trabecular ridges with random orientation and was enclosed either by thin layers of parallel-fibered bone (light purple) or thin layers of osteoid. These observations together with the histomorphometric analysis suggest that the effects of acid bone lysates on bone regeneration are restricted to the area of the collagen membrane.

Discussion

The main finding of the present study was that acid bone lysates lyophilized on collagen membranes reduce bone formation in a critical size defect on rats. Despite the high variation within each group, the impact of acid bone lysate compared to a serum-free medium control was consistent and significant. This observation was somehow unexpected based on the widely held belief that growth factors released by osteoclast during graft resorption support bone regeneration. Certainly, acid treatment of bone chips cannot simulate the complex activity of osteoclasts via a continuous acidification and a simultaneous proteolytic cleavage of the collagen-rich extracellular matrix by the cathepsin K and other proteases.[22] Acid bone lysates can only partially, if at all, represent the osteoclastic activity during the early stage of graft resorption. Nevertheless, there are functional similarities between the acid bone lysate and what is released from osteoclasts. For example, in vivo, TGF-β liberated by osteoclasts recruits mesenchymal cells to the site of bone remodeling.[11] Acid bone lysate, apart from being a rich source of TGF-β, can activate the respective signaling pathways on mesenchymal cells.[9] It should be noted however, that the proposed involvement of TGF-β signaling regarding the attenuating effects of acid bone lysates remains to be examined, for example, by using a pharmacologic inhibition of TGF-β receptor type I kinase such as SB431542.[23]

These findings with acid bone lysates are partially consistent with our earlier observation gathered with bone conditioned medium, which is also a bone-derived aqueous fraction containing TGF-β1[24], leading to a slight reduction of bone formation in a rat calvaria defect model.[19] Since bone conditioned medium is not subjected to acid lysis[24] this previous report may be considered a related control experiment[19]. In vitro, TGF-β signaling dramatically inhibits the BMP-2-dependent calcification, [25] similar to what we have observed with the platelet secretome.[26] The present observations are supported by other reports showing that the canonical TGF-β1 signaling, via smad-3, decreases wound healing in mouse models.[27] This also holds true for fracture healing[28]. In contrast, non-canonical signaling via TGF-β-activated kinase 1 (TAK1) supports cutaneous tissue repair.[29] In this context, it should be mentioned that acid bone lysate is a complex cocktail of 394 proteins, including but not limited to TGF-β1,[6] therefore possible explanations of the findings presented here should not be limited to TGF-β signaling. Notably, bone regeneration is almost restricted to the spongy part of the collagen membrane.[19] Again, one might speculate that bone-derived TGF-β tends to accumulate onto the collagen matrix increasing its concentration thereby attenuating bone formation.

When considering the histologic section and microCT, some of the newly formed bone was located outside of the defect margin and within the space created by membrane and host bone. This has raised the question that this bone formation might be independent of the presence of acid bone lysate adsorbed to the collagen membrane. Apart from the central compartment, where bone regeneration was significantly advanced in the control group compared to acid bone lysate group, two other regions of interest were analyzed, the ectocranial compartment and the outer compartment. Interestingly, acid bone lysate had no impact on bone formation, in these two regions. It can thus be assumed that the impact of acid bone lysate is restricted to the area defined by the collagen membrane that adsorbs TGF-β1 and other molecules serving as an osteoconductive carrier with a retard function. This osteoconductive function is supported by the regeneration pattern displayed on the µCT

images suggesting that collagen membranes are not just passive barriers[30]. However, it cannot be ruled out that acid bone lysates may have modified the structure of the original collagen membrane, particularly after lyophilization and thus having an impact on bone regeneration. From these observations it becomes crucial to determine which proteins and other molecules within the acid bone lysate remain adsorbed to the collagen membranes and are limiting bone regeneration.

The clinical relevance of the present investigation is a matter of speculation but it provides at least a possible explanation why during the resorption phase of graft consolidation bone formation is attenuated.[3] An abundance of TGF-β in the local microenvironment may reduce the migration of mesenchymal cells for coupled bone formation[31]. At physiological levels however, TGF-β1 might trigger the local expression BMP-2 thereby promoting osteogenic differentiation. Some support for this hypothesis comes from our findings that acid bone lysates support the expression of BMP-2 in mesenchymal cells.[9] Based on this theory, it is not the TGF-β released from bone by osteoclasts that initiates osteogenic differentiation, but rather an indirect effect that involves the local expression of BMP-2. Another related aspect is the fact that demineralized bone is osteoinductive, whereas the acid bone lysate has the opposite effect. In this context, what does demineralized matrix retain that promotes bone formation contrary to the suppression in osteogenic differentiation induced by acid bone lysates? One may speculate that the osteoinductive BMPs remain attached to the original bone extracellular matrix and are not released by the acid treatment.[32] Indeed, proteomic analysis of acid bone lysates failed to detect BMPs.[6] From a clinical point of view, it is relevant to understand how autograft resorption is coupled with bone regeneration and how the process is controlled. The present findings might become clinically relevant once it can be shown that the growth factors released from bone during resorption of grafts indeed reduce or even increase bone formation.

The present study has other limitations that should be taken into consideration. First, the present report involved a xenogenic setting, using a porcine peritoneum-derived collagen membrane and porcine acid bone lysate tested on a rat defect model. Second, the collagen membranes underwent lyophilization together with a serum-free medium or the acid bone lysates. Considering that we did not include a regular collagen membrane, care should be taken when interpreting the results regarding the osteoconductive properties of the original collagen membrane. Since TGF-β and presumably also other growth factors adsorb to collagen membranes[10], lyophilization may be avoided in future research. Potential research could focus on the question whether acid bone lysates reduce the formation of h-type endothelial cells that carry the osteogenic progenitors into the defect,[33] or if the migration, proliferation or osteogenic differentiation of osteogenic cells is impaired in vivo. Research is also required to understand which of the numerous proteins in acid bone lysates are responsible for the decrease in bone regeneration and whether the effects of acid bone lysates only occur when loaded onto collagen membranes. The present investigation certainly provides a few answers but raises many other new questions.

Taken together, these findings indicate that bone-derived growth factors comprised in acid bone lysates are able to attenuate bone regeneration in a rat calvaria defect model.

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Figure legends

Figure 1. Micro CT overview of the defect anatomy, bone in contact with host bone and bony islands after 4 weeks of healing. Rat calvaria defects were treated with Bio-Gide membranes either soaked in (A, C, E) serum-free medium, or (B, D, F) in acid bone lysates (ABL). Micro CT pictures representing the samples with (A, B) minimum, (C, D) median and (E, F) maximum bone volume based on quantitative analysis.

Figure 2. Acid bone lysate (ABL) reduces bone regeneration in the calvaria defect. Quantitative analysis of a) bone volume and b) bone defect coverage in the region of interest that is the inner 4 mm diameter of a 5 mm defect. Statistical analysis was based on the data observed with the µCT analysis. The two groups were compared with Mann-Whitney U test. *P*-values are indicated.

Figure 3. Histological overview of the defect anatomy after 4 weeks of healing. Rat calvaria defects were treated with native collagen membranes either soaked in serum-free medium (A, C, E), or in acid bone lysate (ABL) (B, D, F). Histological pictures representing the samples with minimum (A, B), median (C, D) and maximum (E, F) bone volume based on quantitative analysis. The local host calvaria bone demarcates the defect borders and appears in light purple. The newly formed bone stained in dark purple appears in the spongy part of the collagen membranes.

Figure 4. The effect of acid bone lysates is restricted to the central compartment. (A) Histomorphometry on bone volume per tissue volume BV/TV (%) was performed at three regions of interest (ROI); (B, Red ROI) the central compartment within defect margins, (C, yellow ROI) the adjacent ectocranial compartments, and (D, green ROI) the outer compartment on the surface of the host cortical bone. The groups were compared with Mann-Whitney U test. *P*-values are indicated.

Figure 5. Detailed overview on the new bone in the control group (CG) and in the acid bone lysate (ABL) group. Note the characteristic features of immature woven bone indicated by the intense purple stain and the large osteocyte lacunae. The dense art of the membrane is visible in the upper part of control group also showing that new bone grows on the spongy part of the collagen membrane.

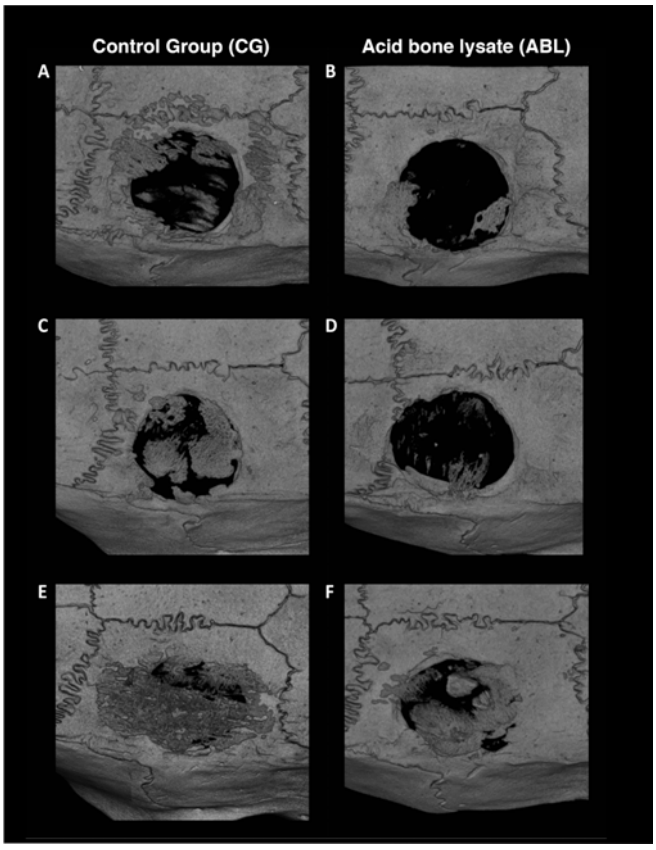

Figure 1

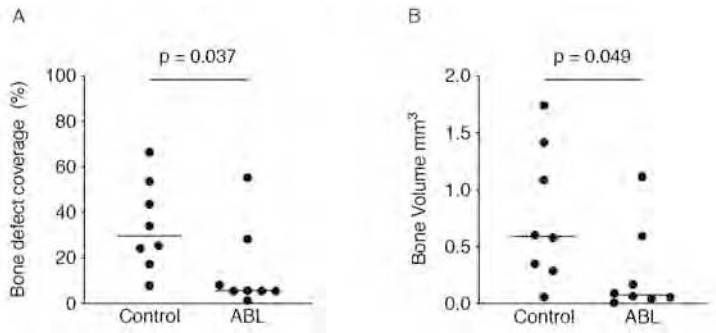

Figure 2

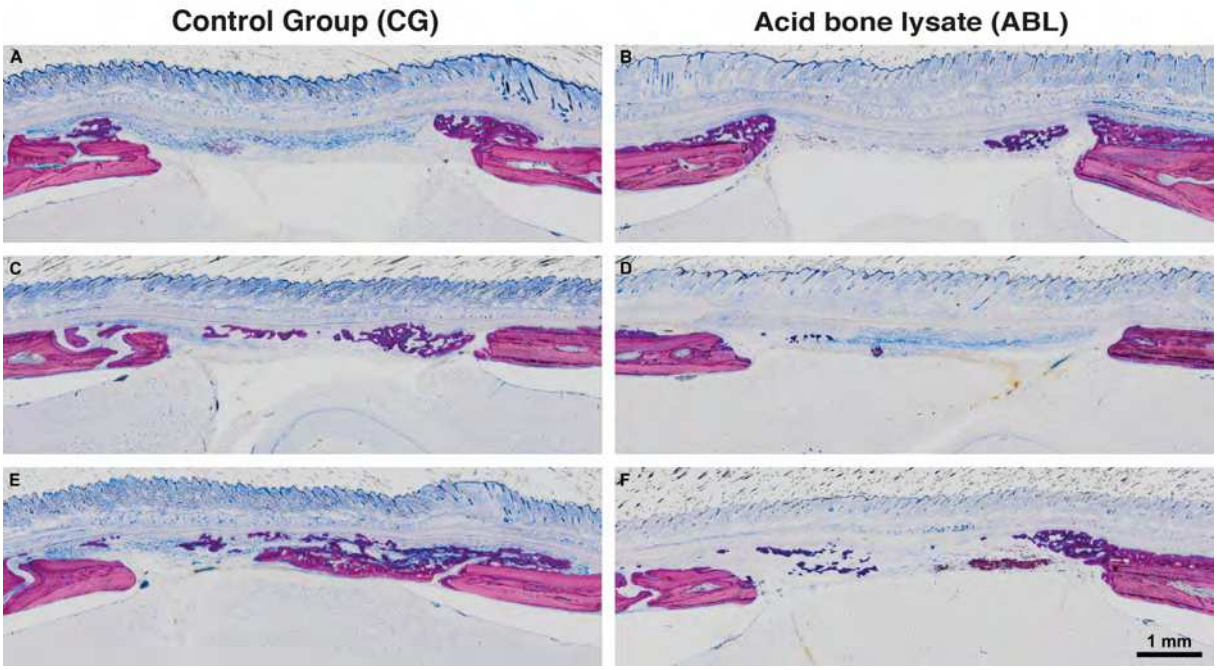

Figure 3

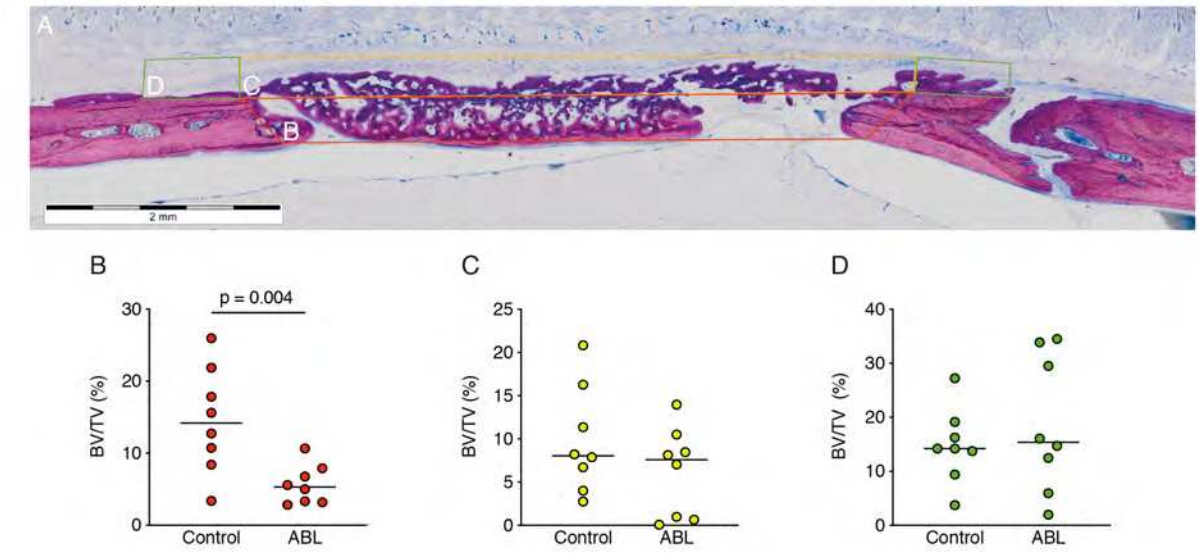

Figure 4

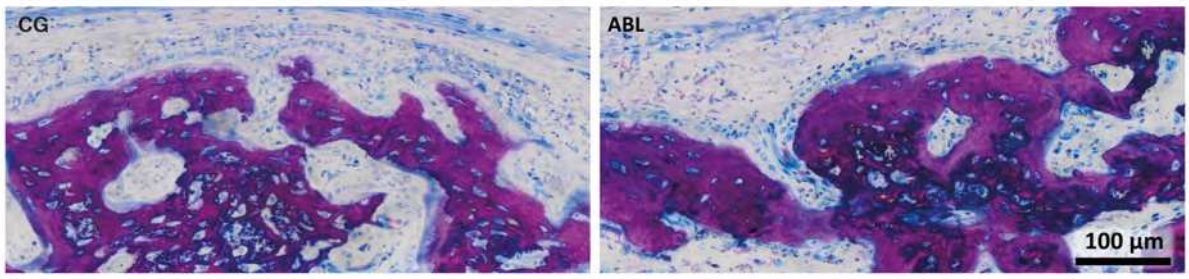

Figure 5