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Biomechanical analysis of engineered bone with anti-BMP2 antibody immobilized on different scaffolds

Sahar Ansari¹, Jin-Ho Phark², Sillas Duarte Jr², Maike Paulino da Silva³, N Sharifzadeh⁴, Alireza Moshaverinia¹, and Homayoun H. Zadeh⁴

¹Center for Craniofacial Molecular Biology, Ostrow School of Dentistry, University of Southern California, Los Angeles, CA

²Division of Restorative Sciences, Ostrow School of Dentistry, University of Southern California, Los Angeles, CA

³Department of Microbiology, Institute of Biomedical Science, University of São Paulo, Sao Paulo, Brazil

⁴Division of Periodontology, Ostrow School of Dentistry, University of Southern California, Los Angeles, CA

Abstract

Recently we have demonstrated the ability of monoclonal antibodies (mAb) specific for bone morphogenetic protein (BMP)-2 immobilized on different scaffolds to mediate bone formation, a process referred to as Antibody Mediated Osseous Regeneration (AMOR). One of the key properties of regenerated bone is its biomechanical strength, in particular in load-bearing areas. This study sought to test the hypothesis that the biomechanical strength of regenerated bone depends of the mode of regeneration, as well as the scaffold used. Four different scaffolds, namely titanium granules (Ti), alginate hydrogel, anorganic bovine bone mineral (ABBM), and absorbable collagen sponge (ACS) were functionalized with anti-BMP-2 or isotype control mAb and implanted into rat critical-size calvarial defects. The morphology, density and strength of the regenerated bone were evaluated after 8 weeks. Results demonstrated that scaffolds functionalized with anti-BMP-2 mAb exhibited varying degrees of bone volume and density. Ti and ABBM achieved the highest bone volume, density and strength of bone. When anti-BMP-2 mAb was immobilized on Ti or ABBM, the strength of the regenerated bone were 80% and 77% of native bone respectively, compared with 60% of native bone in sites implanted with rh-BMP-2. Control interventions with isotype mAb did not promote considerable bone regeneration and exhibited significantly lower mechanical properties. SEM analysis showed specimens immobilized with anti-BMP-2 mAb formed new bone with organized structure bridging the crack areas. Altogether, the present data demonstrated that the morphological and mechanical properties of bone bioengineered through AMOR could approximate that of native bone, when appropriate scaffolds are used.

Corresponding Author: Homayoun H. Zadeh, D.D.S., Ph.D., Associate Professor, Director, Post-Doctoral Periodontology Program, Laboratory for Immunoregulation and Tissue Engineering (LITE), Division of Periodontology, Diagnostic Sciences & Dental Hygiene, University of Southern California, Ostrow School of Dentistry, 925 34th Street Room 4278, Los Angeles, CA 90089-0641, zadeh@usc.edu.

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Keywords

Biomaterials; tissue engineering; mechanical properties; antibody-mediated bone regeneration; Monoclonal antibody; Bone morphogenetic protein

INTRODUCTION

Bone tissue engineering seeks to leverage the biological activities of growth factors and progenitor cells acting on appropriate scaffolds to regenerate bone that matches the physical and biological properties of native bone.^{1,2} Addition of osteoconductive scaffolds is generally sufficient for repair of small intraosseous defects.^{2,3} This is due to the ability of the host reparative mechanisms to provide the required growth factors and progenitor cells that can easily reach the defect site. However, in larger defects, which are larger than the critical size, the inherent reparative capacity of the host is not enough for complete repair.^{4,5} Autologous grafting can treat such defects, due to the fact that autologous grafts provide the progenitor cells, endogenous growth factors and scaffold.¹⁻⁵ However, there are several disadvantages associated with these treatment modalities including: infection donor site morbidity, hematoma, inflammation, pain, and high cost.⁶⁻⁸

To overcome some of these limitations, alternative bone tissue engineering treatment modalities have been proposed, such as gene therapy, stem cell therapy, application of growth factors, or a combination of these strategies. Growth factors such as platelet-derived growth factors (PDGFs) and bone morphogenetic proteins (BMPs) have been used for bone tissue engineering with promising results.⁹ Studies have shown that BMP signaling pathway activates bone formation. These growth factors in this signaling pathway have shown to be potent upregulators of gene and protein expression of osteogenic differentiation and bone formation.¹⁰⁻¹² Among these growth factors, BMP-2, BMP-4 and BMP-7 proteins have been reported to have crucial roles in bone development and repair.^{13, 14} Other studies have shown that osteogenic BMPs such as: BMP-2, BMP-4 and BMP-7 have the ability to stimulate osteoprogenitor differentiation into mature osteoblasts. Both pre-clinical and clinical studies have demonstrated that these BMPs have the osteoinductive potential leading to the FDA approval of recombinant human (rh) BMP-2 and rhBMP-7 as biologic agents used in regenerative medicine and dentistry.¹⁵⁻¹⁸ However, due to several disadvantages, the clinical applications of these osteogenic BMPs are limited.^{19, 20}

An alternative treatment option to administration of exogenous rhBMP-2 is the application of anti-BMP-2 monoclonal antibodies (mAbs) immobilized on a solid scaffold in order to attract endogenous BMP-2, which was first reported by Freire *et al.*, 2011. This approach was termed antibody-mediated osseous regeneration (AMOR).²¹ Freire *et al.* immobilized murine antibodies (mAbs) against BMP-2 on absorbable collagen sponge (ACS) and implanted them within rat calvarial defects.^{21, 22} More recently, we demonstrated the ability of chimeric anti-BMP-2 monoclonal antibodies (mAb) immobilized on different types of scaffold materials to mediate *de novo* bone formation.²³

One of the key properties of any bone is its biomechanical strength, in particular in load-bearing regions. Therefore, it is essential that the biomechanical strength of any regenerated

bone is suitable for its function. To that end, the present study sought to examine the biomechanical properties of bone regenerated by AMOR. It was hypothesized that the mechanical properties of bioengineered bone can vary depending on mode of regeneration, as well as the type of scaffolds used.

EXPERIMENTAL

Antibodies

The hybridoma clone of a murine anti-BMP-2 mAb (3G7, Abnova Inc, Taiwan) was expanded and used in order to fabricate chimeric anti-BMP-2 mAb according to Ansari *et al.*²³

Scaffold biomaterials

Alginate hydrogel (NovaMatrix FMC Biopolymer, Norway), anorganic bovine bone mineral (ABBM) (NIBEC, South Korea), grade IV Porous titanium granules (Tigran Technologies, Malmö, Sweden) and Absorbable Collagen Sponge (ACS, Helicote, Miltex, Plainsboro, NJ) were used in this study. Chimeric anti-BMP-2 mAb (25 µg/mL) and isotype control mAb (25 µg/mL) were immobilized on each scaffold material, as previously described.²³

In vivo calvarial defect model

All animals were treated according to the approved guidelines and regulations from Institutional Animal Care and Use Committee (IACUC) at the University of Southern California. Institutional Animal Care and Use Committee (IACUC) has approved this study (animal protocol number: 20254). Two-month-old virgin female Sprague-Dawley rats (N=72, Harlan Laboratories, Livermore, CA) were used in this study. Critical sized calvarial defects²⁴ were created in the midline of the parietal bone, 7 mm in diameter using a trephine under copious saline irrigation under general anesthesia using xylazine and ketamine. Scaffold materials, each containing 25 µg/mL of mAb, were placed inside the calvarial defects. Next, periosteum was sutured over the calvarial defects to stabilize the biomaterials within defects. ACS scaffold immobilized with rhBMP2 (1.5 mg/ml) was used as the positive control group. At the end of the 8-week treatment period, the animals were sacrificed in a CO₂ chamber and the skulls were harvested and stored in buffered formalin. Eight (n=8) animals were used for each group. Four animals were used in micro-CT/histological studies and four animals were utilized in mechanical testing and subsequent SEM analysis.

Micro-CT analysis

Specimens were scanned using a high-resolution micro-CT system (MicroCAT II, Siemens Medical Solutions Molecular Imaging, Knoxville, TN) for evaluation of ectopic mineralization, with scans of 10 µm width at 60 kV and 110 µA at a spatial resolution of 18.7 µm (Voxel dimension). Bone volume fraction (BV/TV) was calculated. Based on the micro-CT data, the local bone mineral density (BMD) (mg/cm⁻³) of each specimen was analyzed and compared with native calvarial bone using Amira Software (Visage Imaging, Berlin, Germany). Four specimens (n=4) were analyzed for each group. The specimens for micro-CT analysis were used subsequently for the histomorphometric analysis.

Histological and histomorphometric analysis

Half of the retrieved calvarial specimens (four specimens for each group) were fixed with 4% (v/v) paraformaldehyde and prepared according to our previously published protocols.^{23,24} The sectioned specimens were stained with hematoxylin and eosin (H&E). Images were captured using an Olympus DP50 digital camera (Olympus Optical Co, Japan) and the area for new bone formation and the percentages of defect fill were analyzed using NIH ImageJ software by determining the percentage of new bone fill out of the total area. Four specimens (n=4) were analyzed for each group.

Sample preparation and biomechanical testing

The calvaria from remaining animals (n=36) were harvested following euthanasia at 8 weeks post implantation surgery. The implanted biomaterials with a layer of surrounding bone were removed using an 8 mm diameter trephine drill and the host bone surrounding the implants was trimmed with dental burs, leaving a disc-shape specimen. These boney specimens were then rinsed in physiological saline and stored in sterile plastic containers with saline at 4°C to avoid damage to the fragile healed section. The bones were mechanically tested on the day of sacrifice.

The mechanical properties of the regenerated bone was evaluated using a universal mechanical testing machine (Instron, Norwood, MA). The specimens (n=4 specimens/group) were secured in a sample holder by PMMA resin. Mechanical load (compression rate = 0.5 mm min⁻¹) was applied to the center of the former defects in vertical direction by a steel rounded-tip probe with a diameter of 2 mm. During compression, load-deformation values were recorded and stored with the computer software (Bluehill 3 Testing Software) supplied with the testing machine. The center of treated defect sites were subjected to compressive forces exerted at 90° from the probe of a universal mechanical testing machine. The amount of force required to induce mechanical failure (F_{max}) was measured and compared. The maximum load applied to cause fracture of each tested specimen (F_{max}) was determined as a measure of the compressive strength of specimens.^{24, 25} The load – displacement curves were continuously recorded until failure. The mean slope of the linear portion of each curve was calculated as the stiffness of the regenerated bone.³⁰ Four specimens (n=4) were analyzed for each group.

Scanning electron microscopy (SEM)

Following mechanical testing, the topography of the fractured region of each specimen was examined by scanning electron microscopy (SEM; JEOL 5300, Peabody, MA). Briefly, the specimens were washed with PBS at 37°C, submerged in fixative (1% formaldehyde in 0.15 M at pH=7.3) overnight. Specimens were dehydrated using graded alcohol solution and sputter coated with gold. SEM imaging of specimens was conducted at various magnifications.

Statistical analysis of data

Quantitative data were expressed as mean ± standard deviation (SD). A two-tailed Student's t-test and one-way analysis of variance (ANOVA), followed by Tukey's test at significance level of $\alpha = 0.05$, were used for the comparison of multiple sample means. Additionally, the

BMD, BV/TV, and biomechanical data were analyzed with linear regression to evaluate the presence of correlation between the obtained results.

RESULTS

Micro-CT analysis

Micro-CT analysis results are presented in Fig. 1a showing a significant volume of *de novo* bone formation within the calvarial defects implanted with chimeric anti-BMP-2 mAb. In contrast, substitution of the anti-BMP-2 mAb with isotype-matched control mAb lead to lack of significant bone repair. Quantitative analysis of micro-CT results confirmed that sites with anti-BMP-2 mAb on Ti granules and ABBM exhibited the greatest volume of bone (BV/TV), as well as bone density ($P<0.05$). The statistical analysis of data demonstrated that Ti and ABBM scaffolds immobilized with anti-BMP-2 mAb generated significantly greater amounts of bone volume ($P<0.05$) with higher BMD in comparison to the newly regenerated bone by ACS or Alginate scaffolds ($P<0.05$) (Fig. 1b and 1c).

Histological and histomorphometric analysis

Histologic evaluation of repaired calvarial defect sites revealed significant degree of bone fill within defects implanted with each of the four scaffolds pre-loaded with chimeric anti-BMP-2 mAb (Fig. 2a). The presence of vital bone with osteocytes in lacunae was detected with all scaffolds with immobilized anti-BMP-2 mAb (Fig. 2a). In contrast, histologic images of sites treated with scaffolds containing immobilized isotype control mAb were characterized with the presence of connective tissue without appreciable new bone formation (Fig. 2a). Quantitative histomorphometric analysis revealed increased *de novo* bone formation within Ti and ABBM scaffolds, compared to ACS and alginate hydrogel, containing anti-BMP-2 mAb ($p<0.05$) (Fig. 2b). Four specimens were analyzed for each group. The histologic and histomorphometric data were in good correlation with quantitative micro-CT data.

Biomechanical evaluation of regenerated bone

Biomechanical test results demonstrated that for each of the four scaffolds with immobilized anti-BMP-2 mAb, the Fmax was greater than the same scaffold with immobilized isotype-matched control mAb (Fig. 3a). To gain better perspective of the strength of the regenerated bone, the ratio of the Fmax of regenerated bone to that of native bone was calculated. These data revealed that ABBM, Ti, alginate and ACS functionalized with chimeric anti-BMP-2 mAb were able to achieve 77%, 80%, 40% and 28% of the biomechanical strength of native bone, respectively (Fig. 3b). When functionalized with anti-BMP-2 mAb, alginate scaffold showed the lowest degree of mechanical properties ($P<0.05$) followed by ACS scaffold. Comparatively, rhBMP-2 adsorbed on ACS, included as positive control, and achieved 66% of the strength of native bone. The negative control interventions, containing isotype control mAb, did not promote bone regeneration and exhibited significantly lower mechanical properties ($P<0.05$).

Based on the strain-stress curves generated during load to fracture experiments, the stiffness of the engineered bone was calculated. In all the tested samples, an initial linear load-

deformation response was observed (data not shown). However, after the initial phase, a dramatic increase in deformation with a small change in load was observed leading to fracture and failure of the specimens. A significantly higher load to fracture was observed for Ti and ABBM specimens in comparison to alginate and ACS, where the load to fracture decreased markedly. The results showed that the bioengineered bone using Ti and ABBM were significantly ($P<0.05$) more rigid than that mediated by ACS or alginate (Fig. 3c). Additionally, the data confirmed the rigidity of the regenerated bone was dependent on the presence or absence of anti-BMP-2 and the type of the scaffold. A high degree of positive correlation was observed between maximum load to fracture to bone mineral density ($R^2=0.99$) (Fig. 4a), and to bone volume ($R^2=0.95$) (Fig. 4b). Similarly high positive correlation ($R^2=0.95$) was observed between the percentage of bone area based on histomorphometric analysis and maximum load to fracture for the specimens in each group (Fig. 4c) confirming the important role of the utilized biomaterial in the mechanical strength of the bioengineered bone through AMOR.

SEM characterization

To gain further insights into the nature of the regenerated bone, the fractured edges of specimens subjected to biomechanical testing were carefully examined by SEM imaging. The SEM images (Fig. 5) clearly showed that the fracture of bone regenerated by AMOR occurred within the center of *de novo* bone formation. The biomaterial scaffolds used in AMOR were found to have attached tissue and cells, suggesting that the regenerated tissues were strongly adherent on the biomaterial. In contrast, the negative control group did not promote any bone regeneration showing connective tissue formation with unorganized collagen fibrils. In addition, further analysis confirmed that in ACS and alginate specimens, the fractures happened within the regenerated bone (at the site of load application-white arrows) showing the lower mechanical properties of the regenerated bone. In contrast, in the ABBM and Ti groups functionalized with anti-BMP-2 mAb, the fractures were observed farther away from the site of load application and closer to the junction of regenerated bone and the native bone (black arrows). Collectively, these data corroborated that ABBM and Ti contributed to formation of stronger bone with possibility of the integration of newly formed bone to Ti granules or ABBM particles. This suggested that the biomechanical fracture was due to cohesive and not adhesive failure. Since we examined 8-week time point of healing, which is relatively early, one may speculate that the center of the defects contains relatively immature bone.

DISCUSSION

Bone regeneration is necessary in clinical practice aimed at repairing skeletal defects due to numerous conditions including: congenital defects, inflammatory pathology or trauma.^{24, 25} Our recent studies have demonstrated the ability of anti-BMP-2 mAb immobilized on different types of scaffold materials to mediate *de novo* bone formation.^{21, 23} Since all scaffolds onto which anti-BMP-2 mAb have been immobilized mediated *de novo* bone regeneration, the suitability of various scaffolds for translational applications needs to be examined. An important consideration before introduction of bone regenerative modalities into clinical practice is the mechanical properties of the regenerated bone. In particular,

regeneration of load-bearing skeletal bone has to withstand functional loading. It has to be emphasized that significant improvements in bone regeneration capacity and strength of the engineered bone in Ti and ABBM groups are partly due to the biomaterials' properties used itself. Since different types of scaffold materials have been used in the current study, compression test was used to further compare the response of different biomaterials by evaluation of the load to failure. The data demonstrated that the bone bioengineered by AMOR exhibited significantly greater strength and rigidity. The regenerated bone using anti-BMP-2 mAb was significantly stronger in comparison to the one regenerated in the presence of isotype control mAb. The data confirmed that the strength and rigidity of the regenerated bone was dependent on the scaffold. Ti granules and ABBM scaffolds showed the greatest degree of load to fracture and stiffness compared to ACS and alginate. It should be noted that both Ti granules and ABBM are non-resorbable and radiopaque, hence contributing to increased density detected in micro-CT analysis. Sites implanted with ACS and alginate scaffolds functionalized with anti-BMP-2 mAb exhibited reduced density, compared to Ti granules and ABBM. Results demonstrated that the bone regenerated by anti-BMP-2 mAb immobilized on Ti granules and ABBM exhibited significantly higher density in comparison to that of ACS and alginate. The data for bone volume were in good correlation with fraction analysis results. In view of the fact that both Ti granules and ABBM are both radio-opaque, micro-CT images of defect sites implanted with these scaffolds appeared to exhibit high density in groups that were pre-loaded with anti-BMP-2 or isotype control mAb.

The maximum load to fracture was determined, as the force required causing fracture of the repaired bone. Results demonstrated that anti-BMP-2 mAb pre-loaded on titanium and ABBM yielded the highest bone volume, density and mechanical strength. The fact that up to 80% of the strength of native bone was achieved in bone regenerated by AMOR is very significant in clinical applications requiring mechanical loading. Alginate scaffold showed the lowest amounts of mechanical strength ($P<0.05$) followed by ACS scaffold. The negative control groups did not promote any bone regeneration showing significantly lower mechanical strength ($P<0.05$). The greater mechanical properties found with Ti and ABBM reflects the contribution of the scaffold material used. Moreover, the decreased fracture strength of the engineered bone with ACS and alginate scaffolds is possibly attributable to a decrease in the fracture strength of the scaffold material. The SEM images clearly showed specimens immobilized with chimeric anti-BMP-2 mAb formed new bone with organized collagen fibrils bridging the crack areas. In contrast, the negative control interventions did not promote any bone regeneration showing connective tissue formation with unorganized collagen fibrils.

The possible explanation for the abovementioned findings may be attributed to the biomechanical properties (compressive strength) of the biomaterials used. Alginate and ACS are biodegradable and have low compressive strength, whereas, ABBM and Ti are not biodegradable with significantly higher compressive strength. SEM analysis showed a distinct difference between the fracture pattern observed for ACS/Alginate groups compared to Ti and ABBM groups. In ACS and alginate specimens, the fracture happened within the regenerated bone, at the site of load application, confirming the lower mechanical properties of the newly formed bone. On the other hand, the ABBM and Ti groups immobilized with anti-BMP-2 mAb exhibited fracture patterns farther away from the site of load application

that were closer to the junction of regenerated bone and the native bone. This observation might be related to the integration of newly formed bone, with higher strength, to Ti or ABBM or to the compressibility of the biomaterial used.

In the present study, rhBMP-2 with ACS was used as positive control, because it is the most common form of biologic therapy approved by US FDA for specific clinical indications. The clinical applications of rhBMP-2 include repair of non-union tibia fracture, spinal fusion, as well as repair of craniofacial defects.^{1–7} Each of these indications requires high degree of load-bearing for the repaired bone. This mode of bone regeneration achieved 66% of the strength of native bone. Previous studies of rhBMP-2 used in conjunction with other scaffolds have suggested that ACS is the most appropriate scaffold, since the outcomes with ABBM were less favorable.^{26, 27} For this reason, we did not pursue application of rhBMP-2 with other scaffolds.

Based on the outcome of the current study, it can be concluded that each of the scaffolds utilized may be suitable for different clinical situations and applications. For example, titanium is used widely for dental implants, as well as orthopedics for replacement of various joints (hips, knees, etc.). The present results suggest that anti-BMP-2 mAb can potentially be used for improving the osseointegration of titanium to bone.^{28, 31} Due to their physical properties, alginate and ACS may be suitable for intra-osseous defects, where it is desirable to have a biodegradable scaffold and compressive strength will not be required.³² Bioceramics, such as ABBM are likely to have application in repair of osseous defects, which require resistance to compression. Additional biomechanical testing such as compressive, tensile, and nanoindentation testing will be required to provide a more comprehensive understanding of the biomechanical properties of bone bioengineered by different modalities. These studies are currently ongoing in our laboratory and the results will be reported in due course.

CONCLUSIONS

Herein, we report the application of chimeric anti-BMP-2 mAb pre-loaded on different biomaterials to be used as scaffolds in AMOR. We showed that the scaffold used for immobilization of chimeric anti-BMP-2 mAb could affect the biomechanical properties of the regenerated bone. The ability of ABBM and titanium functionalized with chimeric anti-BMP-2 mAb to achieve 77%, and 80% of the biomechanical strength of native bone demonstrated that AMOR is an effective tissue engineering approach with potential application in repair of load-bearing bones. We confirmed the ability of chimeric anti-BMP-2 mAb to functionalize different biomaterial with varying characteristics to mediate osteogenesis. Selection of appropriate scaffold needs to consider the biomechanical requirements of the site being reconstructed.

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Figure 1.

Micro computed tomographic analysis of specimens at 8-weeks post- implantation of different biomaterials used as scaffolds for AMOR. (a) Representative 3-D rendered images following micro-CT scanning of calvarial defects implanted with biomaterials preloaded with chimeric anti-BMP-2 mAb or isotype control mAb. (b) Quantitative analysis of micro-CT data was carried out and expressed as bone volume fraction (BV/TV) for each group (N=4). (c) The bone mineral density (BMD) of each retrieved specimen revealed that sites implanted with anti-BMP-2 mAb immobilized on Ti granules or ABBM exhibited significantly higher density in comparison to sites implanted with anti-BMP-2 mAb immobilized on ACS or alginate. ACS scaffold immobilized with rhBMP2 (1.5 mg/ml) was used as the positive control intervention. *p<0.05, **p < 0.01. NS= not significant.

Figure 2.

Antibody mediated bone regeneration *in vivo* histological analysis. (a) Histological analysis of rat calvarial defects implanted with chimeric anti-BMP-2 mAb immobilized on 4 different types of scaffolds. Scale bar = 1 mm for low magnification images and 50 μ m for histomicrographs in high magnification. (b) Quantitative histomorphometric analysis of newly formed osteoid bone. ACS scaffold immobilized with rhBMP2 (1.5 mg/ml) was used as the positive control group. * $p < 0.05$, ** $p < 0.01$. NS= not significant.

Figure 3.

Mechanical strength of the engineered bone depends on the type of the scaffold utilized. (a) Results of biomechanical evaluation of the regenerated bone using chimeric anti-BMP-2 mAb immobilized on different biomaterials, showing Ti granules and ABBM scaffolds regenerated the strongest new bone in comparison to ACS and alginate. (b) Results of biomechanical evaluation of the regenerated bone in comparison to native bone. ABBM, Ti, alginate and ACS achieved 77%, 80%, 40% and 28% of the biomechanical strength of native bone, respectively. rhBMP-2 used in conjunction with ACS, served as positive control, achieved 66% of the strength of native bone. (c) Stiffness (N/mm) of tested specimens 8 weeks post implantation. * $p < 0.05$, NS= not significant.

Figure 4.

(a) X-Y scatter plot illustrating the correlation between bone mineral density (BMD) and maximum load to fracture for each scaffold. (b) X-Y scatter plot showing the correlation between bone volume fraction (BV/TV) and maximum load to fracture of tested scaffolds. (c) X-Y scatter plot illustrating the correlation between percentage of osteoid bone area based on histomorphometric analysis and maximum load to fracture for each scaffold confirming the important role of the utilized biomaterial in the mechanical strength of the bioengineered bone via AMOR.

Figure 5.

SEM analysis. Representative SEM photomicrographs of the fracture site following mechanical testing showing specimens immobilized with chimeric anti-BMP-2 mAb formed new bone with organized collagen fibrils bridging the crack areas while the negative control group did not promote any bone regeneration. Further analysis confirmed that in ACS and alginate specimens the fracture happened within the regenerated bone (white arrows) while, in the ABBM and Ti groups immobilized with anti-BMP-2 mAb, the fractures were observed closer to the junction of regenerated bone and the native bone (black arrows).