ACTIVATED CARBON AS A BONE SUBSTITUTE: ENHANCING MECHANICAL AND MORPHOLOGICAL PROPERTIES IN A RAT TIBIA DEFECT MODEL.

ABSTRACT

Activated carbon (AC), a highly porous and low-cost material, was tested as a biosubstitute material for healing bone defects. The aim of this work was to investigate the use of 4 different activated carbon materials in the tissue repair process, verifying morphological and biomechanical aspects of the bone. Experiments were performed by drilling rat tibias and filling the resultant bone cavities with four kinds of ACs (AC1, 2, 3 e 4). A Control group (CTL) and untreated lesion group (NT) were also included. The efficiency of the repair was evaluated after 30 days. No alteration of hepatic and renal activity was found, both by histological evaluation of those organs and by the levels of SGOT/SGTP and urea. An increase in ALP levels was observed in the NT group, while all the groups with ACs maintained this enzyme close to the values of the CTL group. The histological study of bone was carried out to evaluate the organization of the formed bone tissue, compared with the quality of repair after treatment. The biomechanical properties (Maximal Force = Fmax and Maximal Deformation = Dmax) of bone were evaluated by three-point flexural tests. The NT group presented immature bone tissue and, although the AC1, AC2 and AC3 groups presented granulation tissue, indicating a delay in bone organization, the Fmax values maintained similar to the NT group. Group AC4 showed mechanical properties and tissue organization similar to the CTL group. Activated carbons allow tissue growth in a rat tibial bone defect model. However, the specific structural characteristics of ACs are important and may contribute to a better organization of bone tissue, since AC4 presented better histological and biomechanical results than AC1, AC2 and AC3 materials. In conclusion, the activated carbon AC4 (Norit ROX0.8) enabled organized bone growth with mechanical properties similar to the normal tissue CTL, in a rat tibial bone defect model. The superior performance of AC4 may be related to its structural characteristics.

Keywords: Activated carbon; biomechanical properties; biosubstitute; bone healing.

1. INTRODUCTION

Bone diseases such as fractures and bone defects are frequent problems met by the worldwide population. Fractures may result from direct or indirect trauma, twisting or falls[1], whereas bone defects can arise from either pathologic processes or orthopaedic problems including trauma, congenital malformations, infections, tumours and surgical excision.[2,3] Those structural bone changes may considerably reduce the quality of human life, initially affecting the locomotion and later presenting serious side effects.

Bone is a complex biocomposite material morphologically consisting of cells and fibres, and basically composed of a collagen matrix, hydroxyapatite micro and nanocrystals ($Ca_{10}(PO_4)_6(OH)_2$ with Ca:P ratios ranging from 1.37-1.87), water and a ground substance such as inorganic salts and proteins.[4,5] A rupture in the continuity of the bone tissue produces a mechanism of repair.

Bone healing is a process of reconstruction involving multiple steps, essentially divided in three different phases: inflammatory, reparative and remodelling.[6,7] The proper functioning of these steps has a great relevance for an effective bone repair. In the first phase, a hematoma is formed within the fracture, caused by the interruption of normal vascular function and distortion of bone architecture.[7,8] The second phase is subdivided in two stages: first a fibrocartilaginous callus is formed, whose size is directly proportional to the patient's movement in the fracture zone, then a formation of irregularly-shaped primary bone callus is established. Finally, the last stage of bone repair is marked by a gradual remodelling towards the original bone shape. The remodelling is performed through a conversion of irregular tissue matrix in bone, a process regulated by resorption followed by bone formation.[7-9] These morphological changes caused by injury are easily evidenced by histological studies.[10,11]

The therapies usually performed in the case of fractures are based on conservative treatments such as complete immobilisation of the affected bone or surgical intervention using implants or biomaterials, whereas the bone defects are corrected either by bone grafting procedures[12] or by implantation of biocompatible prostheses.[13-15] Those current correction processes are costly, partly due to the type of surgery employed but mostly because of the cost of the material used in the repair.[15]

In order to overcome those problems, many former studies showed the potential application of biomaterials[4,16-21] or alternative therapies[22-24] in bone reconstruction, with the purpose of allowing a simpler, faster and cheaper surgery. However some materials still remain expensive. Furthermore, most of the currently employed biomaterials are also based on nanoparticles, and several works have shown that materials containing nanoparticles or nano-sized structures such as hydroxyapatite, titanium dioxide, gold, silica and silver[25-28] may induce toxicity.

The accumulation of hazardous substances on the liver and kidneys causes a great concern, especially when internal morphological and physiological changes appear.[25] Both the liver and the kidney are indeed extremely important organs, acting on the metabolism and the excretion of those agents, respectively. The liver metabolises toxins from the body, whereas the kidneys filter the bloodstream for eliminating toxic substances as exogenous elements, in order to maintain the balance of electrolytes in the body. Thus, investigating kidney and liver functions through the evaluation of either their activity or their morphological changes, or even the accumulation of biomaterials on those organs, is relevant for testing activated carbons (ACs) as potential bone biosubstitutes.

Activated carbons are highly porous solids, generally obtained from low-cost precursors such as biomass, biomass waste or mineral coals by thermal activation at temperatures typically below 1000°C.[29,30,31] According to the gold standards of tissue engineering, an ideal bone substitute should present highly porous three-dimensional structure for ensuring circulation of nutrients and growth factors.[32,33] Thus, the large number of pores of different diameters and the high surface area of ACs make them *a priori* suitable for interacting with various chemicals and serve as facilitators for cell proliferation.

The aim of the present study was to test activated carbons in the bone repair process of rat tibia, and to evaluate the renal and hepatic functions after healing through the quantification of serum glutamic oxaloacetic transaminase (SGOT), serum glutamic-pyruvic transaminase (SGTP) and urea. Phosphatase alkaline levels were evaluated and related to bone formation and morphological changes of bone, kidneys and liver from rats were analysed by histological

studies, while the performance of the biomaterial was evaluated by biomechanical properties of bone after treatment with ACs and comparison with the healthy and non-treated bone.

2. METHODOLOGY

2.1. Ethical aspects

The study procedure was submitted to - and approved by - the local Ethical Research Committee at the University Nove de Julho (Nº 8675271021). All post-surgical care was used to ensure the well-being of the animals, with all rats housed individually and receiving food, water and environmental enrichment. The 3Rs (reduction, refinement and replacement) required by the ethics committee were respected, and 5 animals per group were approved, totalling 30 animals. Surgery to induce bone lesions was performed on both tibias, totalling N=10 tibias per group, distributed in histological or mechanical properties analyses.

2.2. Bone defect

Experiments were carried out with 30 male Wistar rats weighing around 200 g, with ad libitum access to food and water. The animals were manually randomized into six boxes with five animals each, a process carried out by the animal technician responsible for the research institution's animal facility. Animals were anesthetised with ketamine/xylazine at 90/10 mg kg-1, respectively, and then submitted to surgery for making the bone cavity through an experimental model. A surgical incision was performed on the shaved skin, in the post-lateral part of the both tibia, for exposing the tibial bone. Then, a bone defect of 2.5 mm2 pierced the cortical layer of the bone with a motorised drill (1500 rpm; DRILLER®).

Once the injury was achieved, the animals were randomly distributed in 6 groups (N=5), as shown in the following experimental protocol. The bone defect of animals from groups 3 to 6 was filled with enough activated carbon (~1.5 mg for each rat) and then the skin was sutured (Figure 1A). All activated carbons, previously sterilised in autoclave, were mixed with some drops of a sterile isotonic solution (NaCl 0.9%) until the formation of a pasty substance, in order to facilitate the insertion of the biomaterial into the bone defect. The particles size of the activated carbons ranged from 45-180 µm.

Euthanasia was performed 30 days post-induction, using intraperitoneal injections of ketamine (270mg/kg) and xylazine (30mg/kg). Blood and tibia samples were collected and stored in numbered plastic tubes. Biochemical, histological and biomechanical analyses were performed by 2 people participating in the research, without knowledge of the sample or experimental group identification (Figure 1B).

2.3. Experimental groups

Each experimental group was composed of 5 animals and divided in different sets as follows: 1- Control group (CTL) – healthy animals, without any surgical procedure (referred to as C in the following); 2- Non-treated group (NT) – animals with injury, i.e., after the surgical incision for having bone cavity without any treatment (called NT); and 3-6 Treated groups –

animals with injury and treated with various activated carbons (AC1, AC2, AC3 and AC4 – Figure 1A)

2.4. Biomaterials

Four different types of activated carbons (ACs) were used for testing them as biomaterials in bone repair: three were commercial products kindly supplied by Norit, and one was produced in the laboratory by KOH activation of Chinese anthracite at 750°C. Norit PK 1-3, Norit GCN 830 and Norit ROX 0.8 are ACs produced by steam activation of various precursors, NORIT ROX 0.8 having been washed with acid to get a high-purity AC. These materials are used in a broad range of purification applications in pharmaceutical, chemical, food and beverage, and water industries. The lab-made activated anthracite was washed with extreme care, first with 1M HCl, and then with distilled water until the pH of the rinse remained constant and close to 6. After drying in an oven during 24h, a very pure AC material was obtained.[34] The Activated carbons details can be found in Figure 1a, recapitulates label, origin, name, precursor and activation process of the ACs used in this study.

2.5. Biochemical parameters

Before euthanasia, blood was collected from each animal and stocked. After 10 minutes, the blood sample (N=5 per group) was centrifuged at 5 000 rpm during 3 minutes for serum separation and then stored at -80°C for examination. The alkaline phosphatase level in serum was measured with the commercial ELISA kits (WIENER®). Renal and hepatic functions were analysed by biochemical studies. Urea, SGOT and SGPT levels were also determined using commercial ELISA kits (WIENER®).

2.6. Histological studies

After the sacrifice of the animals, the samples (kidneys, the liver and the tibia) were removed from rats for evaluating their morphological alterations. The biological tissue (N=5) were fixed in formalin and prepared according to the histological procedures for their inclusion in paraffin.[35] 5 microns-thick sections of each tissue were obtained by a microtome equipment (LEICA RM 2125 RT) and stained with hematoxylin-eosin for subsequent analysis in an optical microscope and photographed through an Olympus Microscope System (Model CX System 41 – Olympus PM10SP Automatic Photomicrographic System).

2.7. Mechanical properties of bone

For biomechanical analysis, the bone (N=5) was tested immediately after removal in order to avoid any change in its mechanical properties. The length and the diameter of the bone were measured before mechanical test and these values were used for the standardisation of the results. The bones were analysed by three-point flexural tests, using an Intermetric200® testing machine. The loading tests were carried out at a load rate of 6 mm/min until bone rupture. All data of force and displacement were continuously and simultaneously recorded. The maximum stress before rupture was calculated and compared between groups. The Maximum Force (Fmax) and Maximum Deformation (Dmax) at the moment of rupture were determined by the mechanical testing machine, after the loading and unloading tests.

2.8. Statistical Analysis

The data were tabulated in a Microsoft Excel 2010® file, and the Shapiro-Wilk test of normality was performed. As a normal distribution was observed, the data were statistically

evaluated by one-way analysis of variance (ANOVA) followed by post-hoc Tukey-Kramer (GraphPad Prism 5 - GraphPad Software, San Diego California USA). The results were expressed as mean ± standard deviation for all groups, and the values of $p < 0.05$ were considered statistically significant.

3. RESULTS AND DISCUSSION

The results were organized into two sections. In the first section, we present findings related to renal and hepatic function. This includes levels of SGOT and SGPT, indicative of hepatic activity, followed by urea levels, which reflect renal activity. Next, we present histological images of the liver and kidney, qualitatively highlighting the structures of these organs. In the second section, we present the levels of Alkaline Phosphatase and correlate them with certain mechanical properties of the tibia, such as Maximum Force (Fmax) and Maximum Deformation (Dmax) at the moment of bone rupture during the three-point bending mechanical test.

3.1. Evaluation of Renal and Hepatic functions

SGOT/SGPT and urea levels: Biochemical parameters are widely used to evaluate the biological functional activity of liver and kidneys because changes of SGOT/SGPT and urea levels into the blood are important indicators of liver and kidneys damage, respectively. [26,29,36] Hence, for evaluating renal and hepatic functions after the use of ACs as biomaterials, quantitative tests on blood were carried out for all rats and analysed by statistical test, as shown in Figure 2. From such statistical tests, no significant changes in hepatic enzyme activity SGOT/SGPT (Figure 2a, 2b) or urea levels (Figure 2c) were found in groups treated with activated carbons (AC1-AC4), compared to the group control CTL.

Liver and kidney histological analysis: For a better evaluation of the biochemical results, histological studies of liver and kidney were also performed for group CTL and for all treated groups (AC1, AC2, AC3 and AC4). The images allowed observing the integrity of those organs through morphological comparisons. The deposition of activated carbon in those organs was also evaluated, because the presence of exogenous particles in biological tissue may cause an inflammatory response.[26] The histological pictures of liver for all treated groups (AC1-AC4), seen in Figure 3(c,e,g,i), showed hepatic blood vessels with normal arrangement. Furthermore, hepatocytes cells were homogeneously distributed in such tissue, remaining preserved like in group CTL (Figure 3a). Likewise, the functional integrity of the kidneys was similar to the healthy tissue (Figure 3b), whereas glomerulus and Bowman's capsule presented normal shape in the treated groups (Figure 3d,f,h,j). In cases of inflammatory processes, the space around the glomerulus is indeed congested by blood cells and the urea levels may significantly increase.[36]

The hepatic enzyme and urea levels were within the normal limits, suggesting that both renal and hepatic activity remained normal for all treated groups. In other words, the different activated carbons did not seem to induce any toxicity on those organs. Likewise, no morphological alteration such as presence of inflammatory cells, oedema or fibrosis was observed in liver or kidneys by histological analysis of such organs. In the liver, for example, the increase in the number of Küpffer cells may indicate a hepatic inflammation. Such inflammatory process may progress through an increase in the synthesis of collagen, and eventually lead to a liver fibrosis and impaired liver function.[26,36] Additionally, no deposition of activated carbon was found on these two tissues. All these results suggested that the use of the activated carbon as bone biosubstitute material does not induce any toxicity on hepatic or renal functions.

3.2. Osteoconductivity

Alkaline phosphatase level: Alkaline phosphatase (ALP) is an enzyme found in many tissues of the body, including liver, bones, kidneys, intestines and placenta. However, the largest concentrations are in the liver or in bones. During the bone healing process, the level of alkaline phosphatase is an important indicator of bone formation, due to its direct correlation with the gradual deposition of hydroxyapatite on the extracellular matrix of bone.[37,38] The function of alkaline phosphatase on the bone healing process is based on the hydrolysis of pyrophosphate, providing inorganic phosphate and contributing to mineralization.[39] Consequently, the deposition of hydroxyapatite affects the activity of osteocytes as well as deposition and bone resorption phases. Normally, the injurious process is characterised by a gradual increase of alkaline phosphatase levels, reaching the maximum peak at the end of the second week. Then, the activity of ALP is gradually reduced and followed by the remodelling phase of bone, which is characterised by the transition of immature to mature bone.[23]

The bone alkaline phosphatase activity was measured and compared between groups. Alkaline phosphatase levels are presented in 4 for all treated groups (AC1, AC2, AC3, AC4), and statistically no significant changes were found compared to group CTL. However, those groups showed lower levels of alkaline phosphatase by comparison with group NT. Increased levels of ALP in the NT group suggest an increase in bone formation activity.[40] The same was not observed in AC groups, which may indicate that the four groups with materials were in a more advanced phase of bone repair.

Bone histological studies: The histological images of bone evidenced distinct characteristics for each group. As expected, the control group CTL (Figure 5a) presented cortical and medullary layers completed with well-distributed osteocytes (mature bone cells), in addition to typical endosteum and vascular channels.

The groups with bone defect, treated and non-treated, presented transition areas of bone formation at different degrees. In general, NT, AC1, AC2, AC3 and AC4 showed well-defined medullary and cortical regions delimited by endosteum cells. Moreover, a homogeneous distribution of osteocytes in the region of bone formation in NT, AC1 and AC4 groups was clearly seen, representing the organization of the bone tissue. On the other hand, the group AC4 presented more complex structures such as vascular channels, also observed in healthy tissue of group CTL, whereas the images of NT and AC1 groups, (Figure 5b, 5c) suggested that these structures were in formation.

The presence and organization of vascular channels are normally formed in more advanced stages of repair, and their function are to provide nourishment to the bone tissue and to remove dead cells. The absence of vascular channels may induce cell death and affect the bone tissue growth.[41] Hence, the presence of vascular channels in the zone of the injury is also an indicator of improved bone repair in the group AC4. The presence of some osteoclasts was detected in NT, AC1, AC2 and AC4 groups. These cells are important in various phases of repair, especially in bone remodeling, because it resorbs the hard callus formed during the reparative phase of the bone healing process.[7]

The group AC4 also showed the lowest transition area and was the only group to present mature bone, indicating progress in bone repair compared to the others groups. Gradually, extensive transition areas were observed in AC1 followed by AC2 and AC3 groups. Moreover, the latter two groups also exhibited an important amount of activated carbon deposited between the transition areas. Transition areas are normal during bone healing and indicate the progression of the repair according to the character of the organization: the presence of mature

bone indicates the advanced stage of the remodeling phase while the granulation tissue is normally found before the remodeling phase.[7,42] The bone histological information obtained in the present study showed that the biomaterial used in the AC1 and AC4 groups promoted more regular aspects in cells and structures, presenting a progress in bone repair through the presence of a more organized bone tissue, whereas the response for AC2 and AC3 was different because the healing process was slower.

Biomechanical properties:

An important factor for using a biomaterial as a bone substitute is the quality of the bone formed during the healing process. When consider the mechanical properties of the newly formed bone, the rearrangement, the bone mass and the thickness of the cortical region, have a direct impact on the bone resistance.[24,43,44] Thus, evaluation of the mechanical properties of these biological tissues woild be an important tool important for understanding this repair process.[45,46]

The performances of all the biomaterials used in this work were evaluated by mechanical properties using the three-point flexural tests of bone after 30 days of healing. The data of Fmax related to stress and Dmax with strain were obtained and analyzed together with the biochemical and histological data

Figure 6(a) illustrates the Fmax values of bone calculated from the maximum force in rupture for all studied groups. The non-treated group NT presented the smallest Fmax value, in comparation with CTL group. These results of mechanical properties added to the biochemical and histological analyses create a profile of the NT group, that presented elevated values of ALP (Figure 4), less histological organisation, and reduced mechanical properties (Figure 6). Normally, during the bone healing process, the injured tissue is slowly regenerated and most of its properties are restored.[47] However, eventual changes in bone microarchitecture and a decrease of bone mineral density may happen and consequently may induce more fragile structures.25,[48]

The groups of rats treated with activated carbons (AC1, AC2 and AC3) did not show any difference in mechanical properties when compared to the NT group, besides presented ALP levels similar to the CTL group. Considering that the NT group simulates normal bone growth after injury, values similar to this group can be considered positive, leading us to consider that carbon material AC1, 2 and 3 also presented important results for bone repair, since they did not harm normal bone growth. However, when analyzing only the histological results, related to tissue organizationit was observed that the AC3 group presented a more disorganized tissue, with the presence of granulation tissue, suggesting a delay in bone healing, when compared with the other experimental groups.

In contrast, the group AC4 presented increase of Fmax values of Fmax compared to the NT, AC1, AC2 and AC3 groups, with values similar to the CTL group. The group of rats treated with these activated carbons (AC4) presented improved bone repair, observed by a series of dates: the normal levels of ALP (see Figure 4), a better histological organisation in comparison to the groups NT and AC1, 2 and 3 (Figure 5) in the region of bone healing, and good biomechanical properties in comparison to the group NT (see Figure 6), presenting bone repair similar to the CTL group, in which was no induction of bone injury. This finding was corroborated by the presence of vascular channels and mature bone tissue, seen through histological evaluation of the injured zone (Figure 5), and were in agreement with the good mechanical properties of group AC4, since those structures indicate a good evolution of bone repair.[49]

Figure 6(b) shows the relationship between Fmax and Dmax, also considered as stress X strain values for the different groups. No differences were observed in bone deformation capacity when comparing all groups. In general, the activated carbon materials used in this study showed promising results in the bone repair process. While materials AC1, 2 and 3 showed similar repair to the NT group, material AC4 proved to be even better, with a repair process similar to the CTL group. Small differences in response between the materials used in this study, regarding tissue organization or the mechanical properties of the formed bone, could be the subject of research in future studies, comparing their use in different bone tissues, where the proportion between the cortical and medullary layers would be different.

The activated carbons used in the present study presented good responses as biosubstitute materials in bone healing. First, no alteration of hepatic and renal activity was found through the observation of three parameters: (1) the histological cells and structures of the liver and the kidneys remained completely preserved, (2) no deposition of ACs on such organs was found, and (3) the levels of SGOT/SGTP and urea were within the normal limits. Second, all studied ACs presented good osteoconductivity properties since the formation of an osseous tissue was always observed, followed by normal levels of ALP in the blood of the treated groups. Moreover, all groups of rats treated with ACs showed either similar or superior histological organisation in the region of bone healing and good biomechanical properties in comparison to the NT group. All data provided here evidenced that the biomaterial AC4 seems to present the necessary characteristics for promoting an efficient repair for the kind of bone investigated in this work.

Although ALP levels are related to bone formation, there are other markers that could strengthen this hypothesis regarding the differences in the repair phases between the NT group and the groups with carbon materials. It would be interesting to evaluate Acid Phosphatase levels, as well as verify the expression of Interleukin 6 and its relationship with Hank-L and bone morphogenetic proteins (BMPs) associated to osteoclast activity in the bone remodeling process.[50] Both the information on tissue organization, obtained from histological analyses, and bone strength, obtained from biomechanical results, are complementary data that make sense when observed together. However, it would be important to investigate the behavior of the carbon materials used in this study on the bone repair process in different bones, where the proportion between cortical and medullary layers differs, significantly altering the mechanical properties of these tissues. Equally important would be the study of the effect of these activated carbon materials during the healing process at different time points, considering both the acute and chronic phases of bone repair.

4- CONCLUSION

The use of activated carbon material as a bone substitute appears to be advantageous and safe, with no signs of renal or hepatic alteration after 1 month of application in the tibia of rats. The AC4 carbon material promoted a best bone repair demonstrated by the formation of tissue with morphological and biomechanical characteristics similar to intact bone.

The use of ACs as a bone biosubstitute is advantageous because such biomaterials are usually derived from sustainable resources with important characteristics such as the connectivity between its pores and low cost. Besides, ACs may be easily produced from several biomass wastes at large scale.

Additional studies with bone cell cultures are important for evaluating cell growth and viability, as well as assessing the characteristics of the carbon material itself, particularly the size and

proportion of pores and the surface properties of these materials, which could support their use in different biological tissues.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that they have no known competing financial interests OR non-financial interests OR personal relationships that could have appeared to influence the work reported in this paper.

ETHICAL APPROVAL

The study was approved by the Ethics Committee on the Use of Laboratory Animals under protocol No. 8675271021. The study involves the use of 30 male Wistar rats and includes bone surgery for the application of the biomaterials under investigation. All surgical and post-surgical care adhered to CEUA guidelines. The doses of anesthetics and analgesics used in the study, as well as measures to ensure the animals' welfare, were carefully followed. The entire experiment was supervised by a veterinarian responsible for the university's animal facility.

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee"

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Authors hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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(A)

Label	Origin	Name	Precursor	Activation method
AC-1	Lab-made	AC - 30	anthracite	KOH+acid/water washed
AC-2	Commercial	Norit PK 1-3	peat	steam
AC-3	Commercial	Norit GCN 830	selected coals	steam
AC-4	Commercial	Norit ROX 0.8	coal	steam+acid washed

(B)

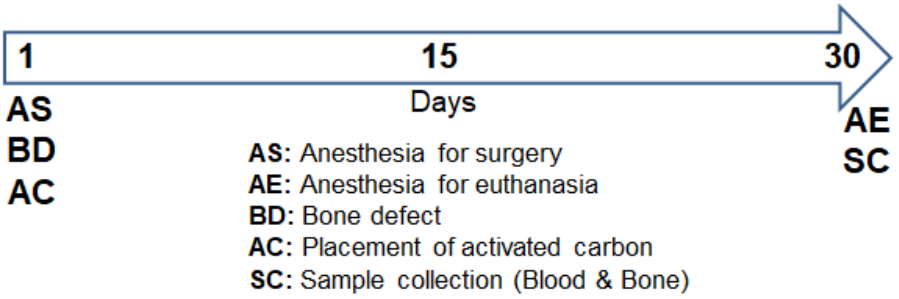


Figure 1: Experimental protocol. In (a) characteristics of the activated carbon materials. In (b) Experimental time course for the bone defect protocol (BD), treatments (AC), Anesthesia (AS), euthanasia (AE) and collection of materials for analysis (SC).

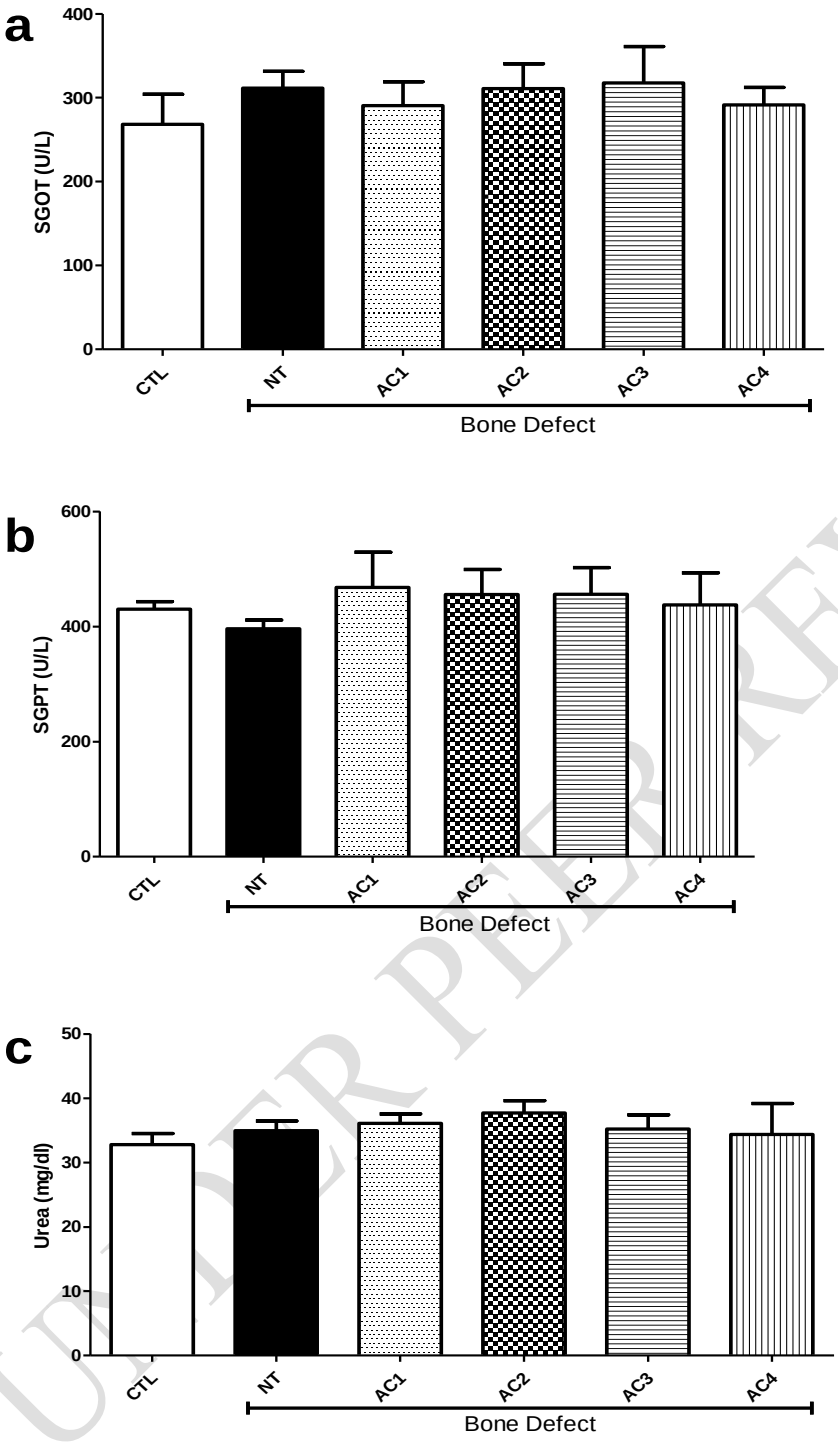


Figure 2. Biochemical analysis of liver and kidney activity. In (a) Serum Glutamate Pyruvate Transaminase - SGOT. In (b) Serum Glutamate Pyruvate Transaminase - SGPT. In (c) Urea Levels. Experimental Groups: CTL: Control without bone defect; NT with bone defect without treatment or treated with carbon materials (AC1, AC2, AC3 and AC4). Statistical test did not demonstrate significant differences between the experimental groups.

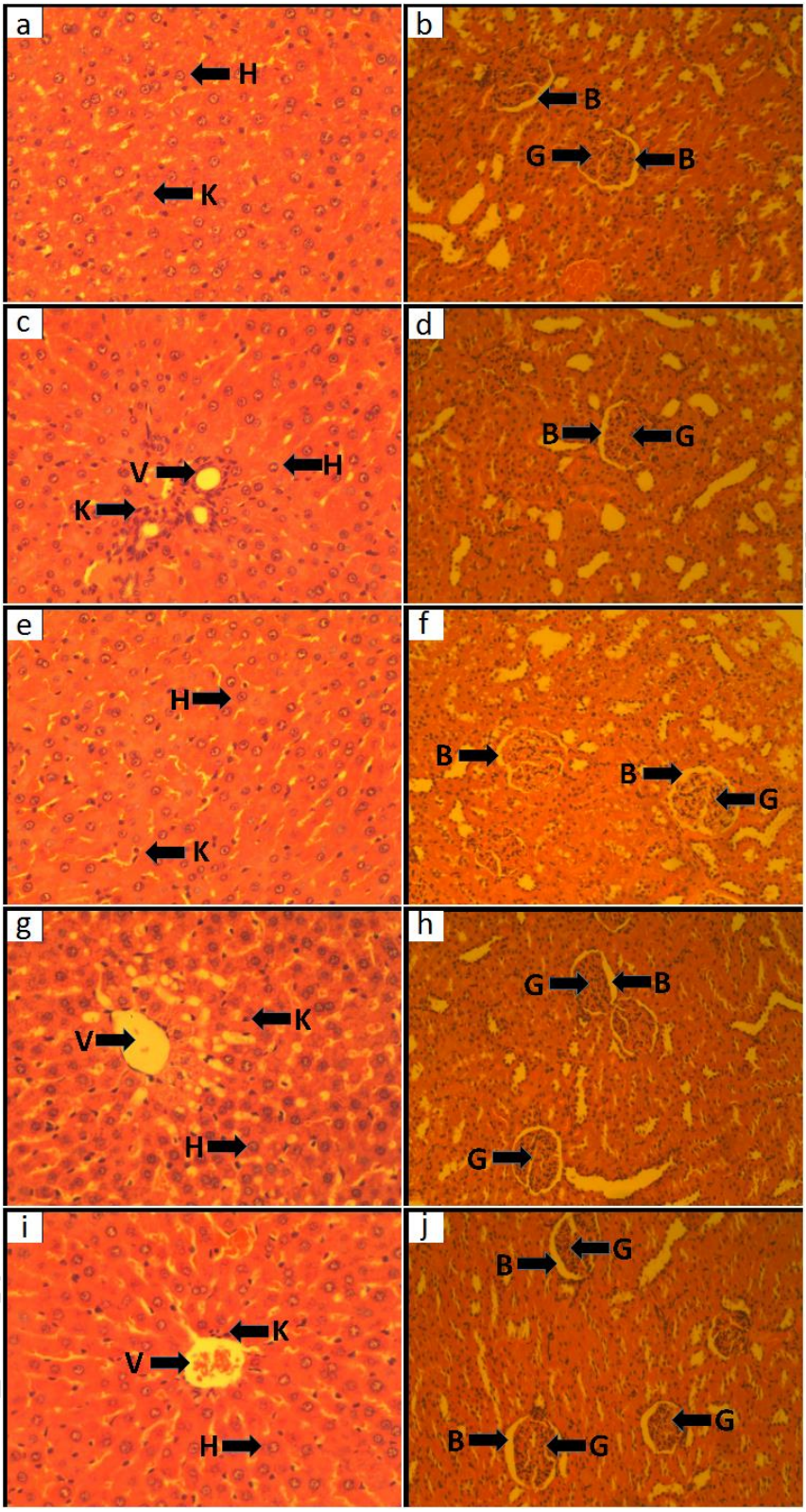


Figure 3. Representative histological images (100X) of liver (left) and kidney (right) tissues, respectively. Groups: CTL (a,b); AC1 (c,d); AC2 (e,f); AC3 (g,h) and AC4 (i,j). Legend: K - Küpffer cells; H - hepatocyte cell; V - blood vessel; B - Bowman's capsule; G - Glomerulus.

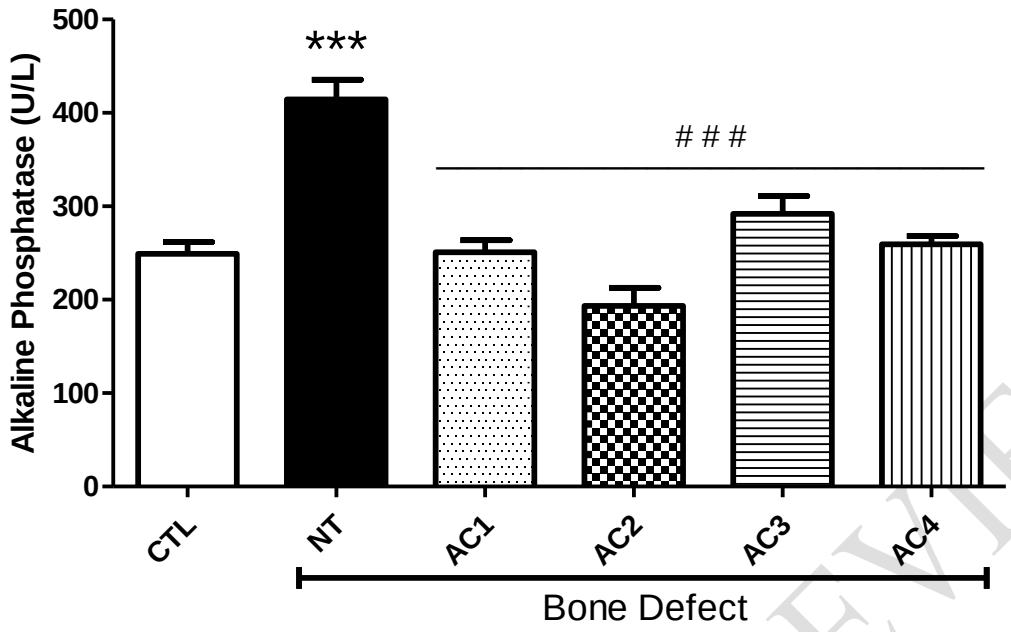


Figure 4. Alkaline phosphatase level - ALP. Experimental Groups: CTL: Control without Bone Defect; NT with defect without treatment or treated with carbon materials (AC1, AC2, AC3 and AC4). *** $p<0.001$ Vs CTL and ### $p<0.001$ Vs NT.

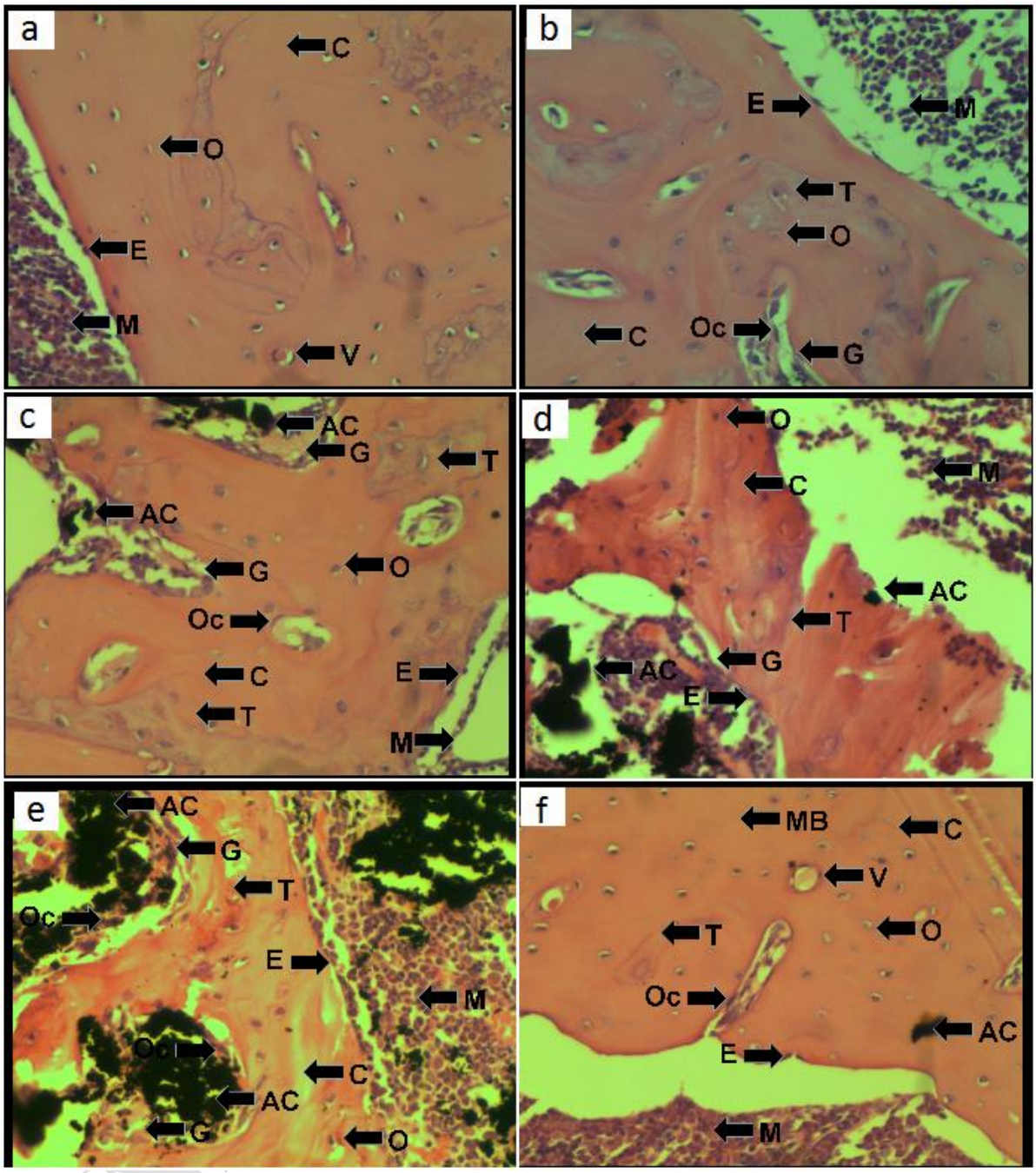


Figure 5. Representative histological images (100X) of the healed region of rat tibia. Groups: CTL (a); NT (b); AC1 (c); AC2 (d); AC3 (e) and AC4 (f). Cortical Region (C); Medullary Region (M); Osteocytes (O); Vascular Channels (V); Osteoclasts (Oc); Activated Carbon (AC); Endosteum (E); Transition area (T), Granulation tissue (G), Mature Bone (MB).

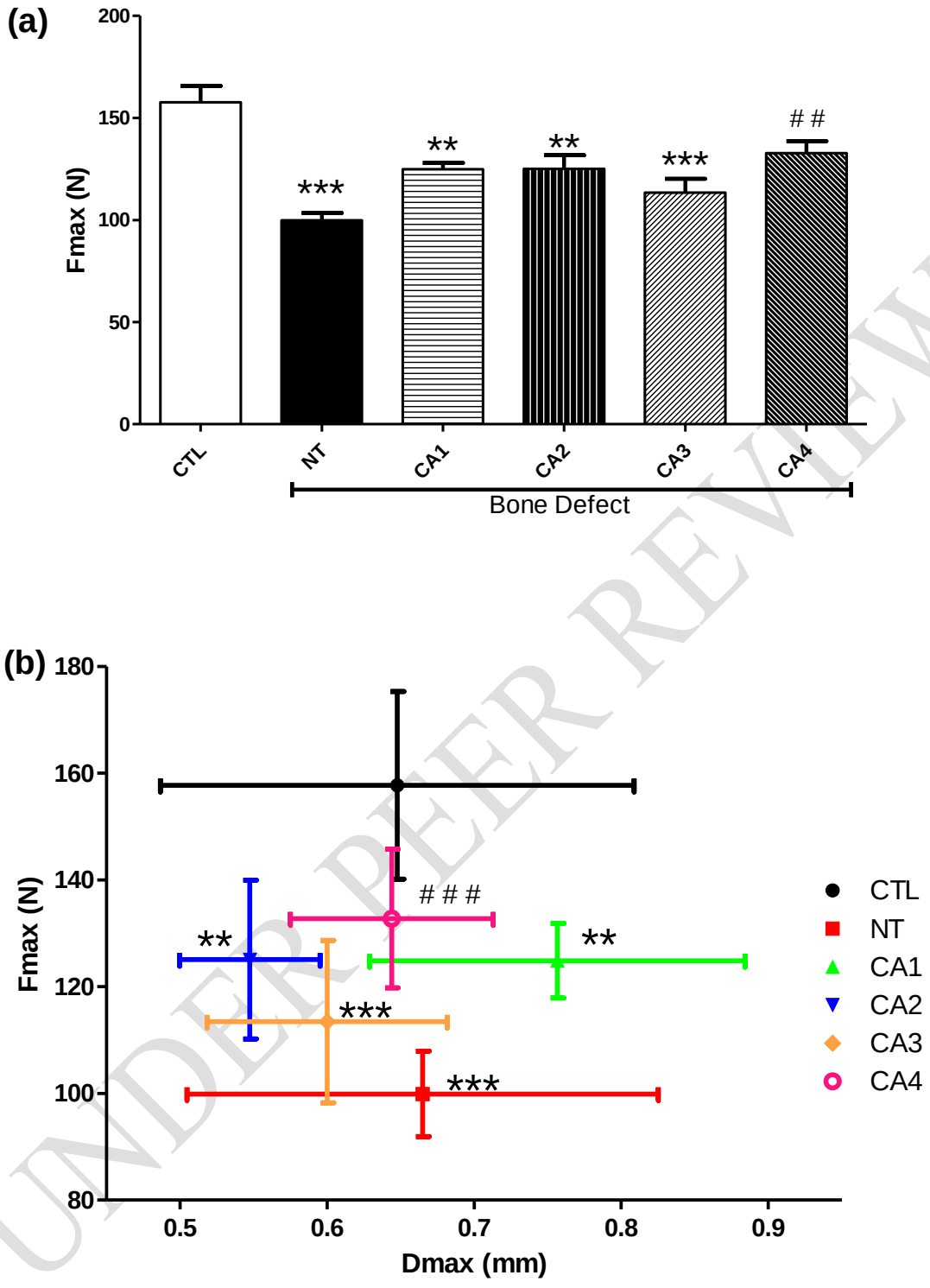
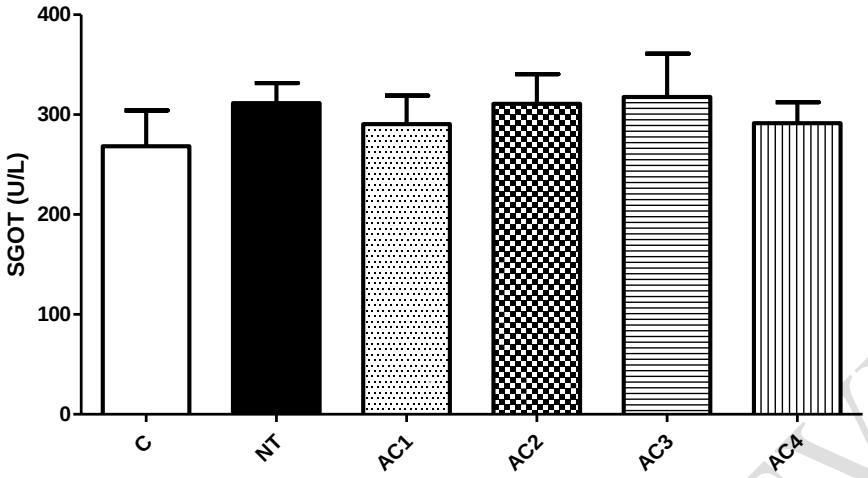
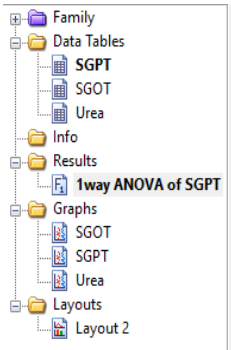


Figure 6. Biomechanical properties from three-point flexural tests. (a) Fmax (Maximum Rupture Force). (b) Relationship between Fmax and Dmax (Maximum Rupture Deformation). Experimental Groups: CTL: Control without bone defect; NT with bone defect without treatment or treated with carbon materials (AC1, AC2, AC3 and AC4). ** $p<0.01$ and *** $p<0.001$ Vs CTL; ### $p<0.05$ Vs NT.

APPENDIX

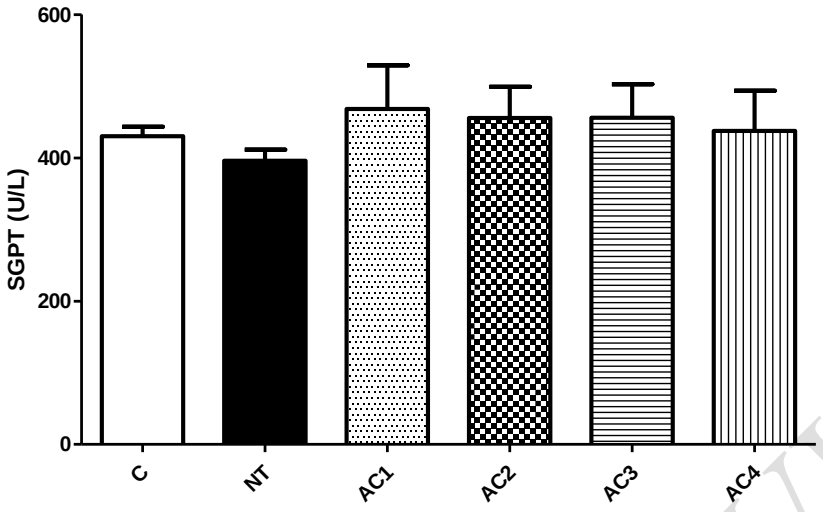
Attachement I: Print screen of the statistical analysis of SGOT – Figure 2A





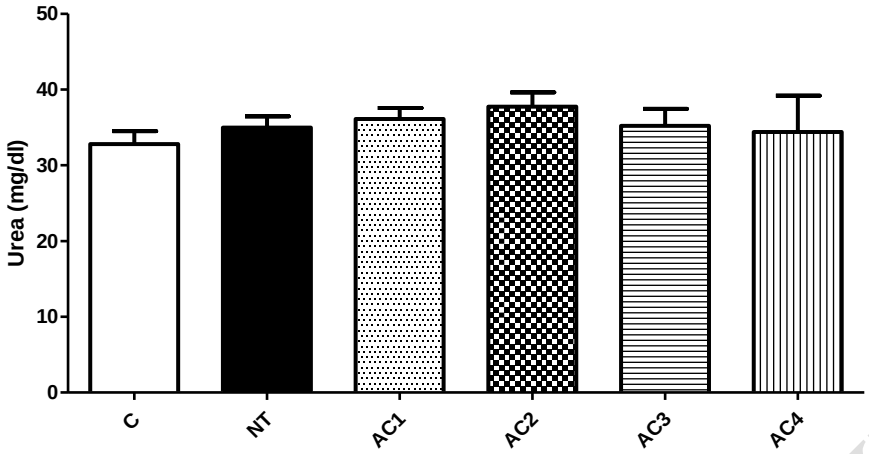
	1way ANOVA					
7	Number of groups	6				
8	F	1.757				
9	R squared	0.2680				
10						
11	Bartlett's test for equal variances					
12	Bartlett's statistic (corrected)	11.43				
13	P value	0.0435				
14	P value summary	*				
15	Do the variances differ signif. (P < 0.05)	Yes				
16						
17	ANOVA Table	SS	df	MS		
18	Treatment (between columns)	16650	5	3330		
19	Residual (within columns)	45490	24	1895		
20	Total	62140	29			
21						
22	Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
23	C vs NT	34.30	1.762	No	ns	-50.83 to 119.4
24	C vs AC1	-37.87	1.945	No	ns	-123.0 to 47.27
25	C vs AC2	-25.49	1.309	No	ns	-110.6 to 59.65
26	C vs AC3	-25.61	1.315	No	ns	-110.7 to 59.53
27	C vs AC4	-7.207	0.3702	No	ns	-92.35 to 77.93
28	NT vs AC1	-72.18	3.707	No	ns	-157.3 to 12.96
29	NT vs AC2	-59.79	3.071	No	ns	-144.9 to 25.35
30	NT vs AC3	-59.91	3.077	No	ns	-145.0 to 25.23
31	NT vs AC4	-41.51	2.132	No	ns	-126.6 to 43.63
32	AC1 vs AC2	12.39	0.6362	No	ns	-72.75 to 97.52
33	AC1 vs AC3	12.27	0.6300	No	ns	-72.87 to 97.40
34	AC1 vs AC4	30.66	1.575	No	ns	-54.47 to 115.8
35	AC2 vs AC3	-0.1200	0.006165	No	ns	-85.26 to 85.02
36	AC2 vs AC4	18.28	0.9388	No	ns	-66.86 to 103.4
37	AC3 vs AC4	18.40	0.9450	No	ns	-66.74 to 103.5

Attachement II: Print screen of the statistical analysis of SGPT – Figure 2B



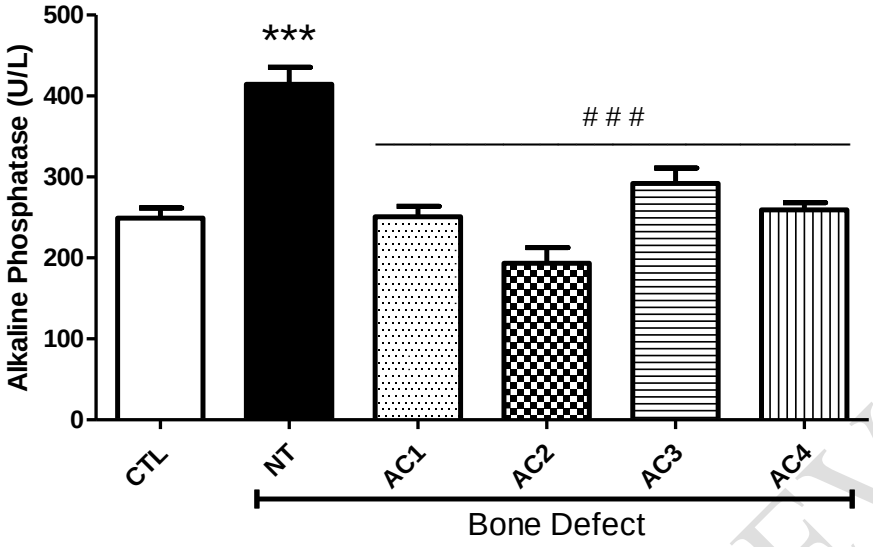
	1way ANOVA					
7	Number of groups	6				
8	F	1.790				
9	R squared	0.2717				
10						
11	Bartlett's test for equal variances					
12	Bartlett's statistic (corrected)	3.201				
13	P value	0.6691				
14	P value summary	ns				
15	Do the variances differ signif. (P < 0.05)	No				
16						
17	ANOVA Table	SS	df	MS		
18	Treatment (between columns)	8532	5	1706		
19	Residual (within columns)	22870	24	953.1		
20	Total	31410	29			
21						
22	Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
23	C vs NT	-43.18	3.128	No	ns	-103.6 to 17.19
24	C vs AC1	-22.06	1.598	No	ns	-82.43 to 38.32
25	C vs AC2	-42.32	3.065	No	ns	-102.7 to 18.06
26	C vs AC3	-49.20	3.564	No	ns	-109.6 to 11.17
27	C vs AC4	-22.98	1.664	No	ns	-83.35 to 37.40
28	NT vs AC1	21.12	1.530	No	ns	-39.25 to 81.50
29	NT vs AC2	0.8665	0.06276	No	ns	-59.51 to 61.24
30	NT vs AC3	-6.019	0.4360	No	ns	-66.39 to 54.36
31	NT vs AC4	20.20	1.463	No	ns	-40.17 to 80.58
32	AC1 vs AC2	-20.26	1.467	No	ns	-80.63 to 40.12
33	AC1 vs AC3	-27.14	1.966	No	ns	-87.52 to 33.23
34	AC1 vs AC4	-0.9203	0.06666	No	ns	-61.30 to 59.45
35	AC2 vs AC3	-6.886	0.4987	No	ns	-67.26 to 53.49
36	AC2 vs AC4	19.34	1.401	No	ns	-41.04 to 79.71
37	AC3 vs AC4	26.22	1.899	No	ns	-34.15 to 86.60
38						

Attachement III: Print screen of the statistical analysis of Urea – Figure 2C



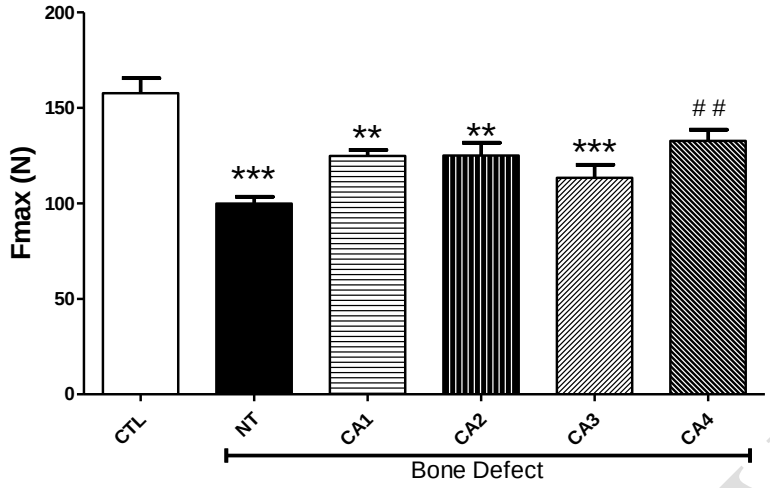
	1way ANOVA					
7	Number of groups	6				
8	F	2.134				
9	R squared	0.3078				
10						
11	Bartlett's test for equal variances					
12	Bartlett's statistic (corrected)	9.464				
13	P value	0.0919				
14	P value summary	ns				
15	Do the variances differ signif. (P < 0.05)	No				
16						
17	ANOVA Table	SS	df	MS		
18	Treatment (between columns)	68.72	5	13.74		
19	Residual (within columns)	154.5	24	6.439		
20	Total	223.3	29			
21						
22	Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
23	C vs NT	-2.192	1.932	No	ns	-7.155 to 2.770
24	C vs AC1	-3.325	2.930	No	ns	-8.288 to 1.637
25	C vs AC2	-4.936	4.350	No	ns	-9.899 to 0.02647
26	C vs AC3	-2.408	2.122	No	ns	-7.371 to 2.555
27	C vs AC4	-1.592	1.403	No	ns	-6.555 to 3.370
28	NT vs AC1	-1.133	0.9984	No	ns	-6.096 to 3.830
29	NT vs AC2	-2.744	2.418	No	ns	-7.706 to 2.219
30	NT vs AC3	-0.2156	0.1900	No	ns	-5.178 to 4.747
31	NT vs AC4	0.6000	0.5287	No	ns	-4.363 to 5.563
32	AC1 vs AC2	-1.611	1.419	No	ns	-6.573 to 3.352
33	AC1 vs AC3	0.9174	0.8084	No	ns	-4.045 to 5.880
34	AC1 vs AC4	1.733	1.527	No	ns	-3.230 to 6.696
35	AC2 vs AC3	2.528	2.228	No	ns	-2.434 to 7.491
36	AC2 vs AC4	3.344	2.947	No	ns	-1.619 to 8.306
37	AC3 vs AC4	0.8156	0.7187	No	ns	-4.147 to 5.778
38						

Attachement IV: Print screen of the statistical analysis of Alkaline Phosphatase – Figure 4



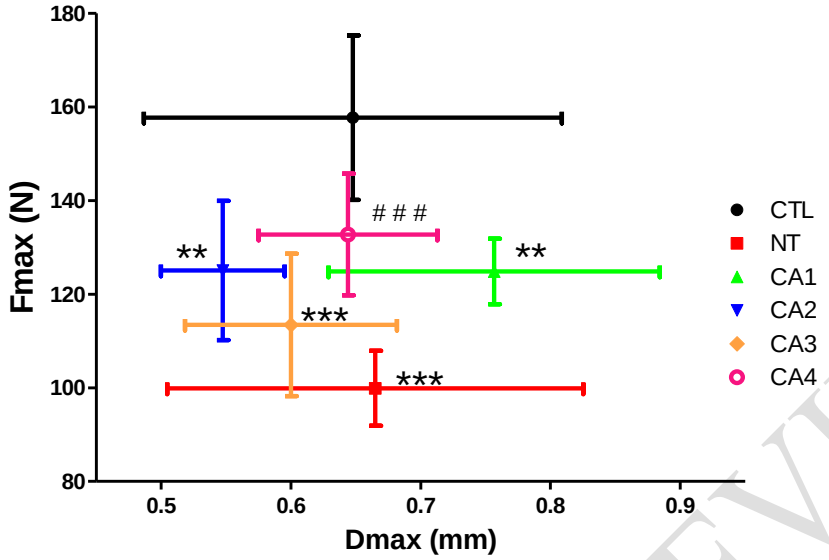
	1way ANOVA					
7	Number of groups	6				
8	F	21.06				
9	R squared	0.8144				
10						
11	Bartlett's test for equal variances					
12	Bartlett's statistic (corrected)	3.710				
13	P value	0.5918				
14	P value summary	ns				
15	Do the variances differ signif. (P < 0.05)	No				
16						
17	ANOVA Table	SS	df	MS		
18	Treatment (between columns)	139700	5	27940		
19	Residual (within columns)	31830	24	1326		
20	Total	171500	29			
21						
22	Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
23	CTL vs NT	-165.4	10.15	Yes	***	-236.6 to -94.13
24	CTL vs AC1	-1.571	0.09646	No	ns	-72.80 to 69.65
25	CTL vs AC2	56.03	3.440	No	ns	-15.20 to 127.3
26	CTL vs AC3	-42.66	2.619	No	ns	-113.9 to 28.57
27	CTL vs AC4	-10.24	0.6284	No	ns	-81.46 to 60.99
28	NT vs AC1	163.8	10.06	Yes	***	92.56 to 235.0
29	NT vs AC2	221.4	13.59	Yes	***	150.2 to 292.6
30	NT vs AC3	122.7	7.533	Yes	***	51.47 to 193.9
31	NT vs AC4	155.1	9.524	Yes	***	83.89 to 226.3
32	AC1 vs AC2	57.60	3.537	No	ns	-13.62 to 128.8
33	AC1 vs AC3	-41.09	2.523	No	ns	-112.3 to 30.14
34	AC1 vs AC4	-8.664	0.5319	No	ns	-79.89 to 62.56
35	AC2 vs AC3	-98.69	6.059	Yes	**	-169.9 to -27.47
36	AC2 vs AC4	-66.27	4.068	No	ns	-137.5 to 4.960
37	AC3 vs AC4	32.43	1.991	No	ns	-38.80 to 103.7

Attachement V: Print screen of the statistical analysis of Fmax – Figure 6A



	1way ANOVA					
1	Table Analyzed	Fmax (N)				
2						
3	One-way analysis of variance					
4	P value	< 0.0001				
5	P value summary	***				
6	Are means signif. different? (P < 0.05)	Yes				
7	Number of groups	6				
8	F	10.87				
9	R squared	0.6937				
10						
11	Bartlett's test for equal variances					
12	Bartlett's statistic (corrected)	4.351				
13	P value	0.5001				
14	P value summary	ns				
15	Do the variances differ signif. (P < 0.05)	No				
16						
17	ANOVA Table	SS	df	MS		
18	Treatment (between columns)	9463	5	1893		
19	Residual (within columns)	4179	24	174.1		
20	Total	13640	29			
21						
22	Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
23	CTL vs NT	57.83	9.799	Yes	***	32.02 to 83.63
24	CTL vs CA1	32.86	5.568	Yes	**	7.053 to 58.67
25	CTL vs CA2	32.65	5.532	Yes	**	6.839 to 58.45
26	CTL vs CA3	44.29	7.505	Yes	***	18.48 to 70.10
27	CTL vs CA4	24.97	4.231	No	ns	-0.8373 to 50.78
28	NT vs CA1	-24.97	4.231	No	ns	-50.77 to 0.8381
29	NT vs CA2	-25.18	4.267	No	ns	-50.99 to 0.6239
30	NT vs CA3	-13.54	2.294	No	ns	-39.34 to 12.27
31	NT vs CA4	-32.86	5.568	Yes	**	-58.67 to -7.053
32	CA1 vs CA2	-0.2142	0.03630	No	ns	-26.02 to 25.59
33	CA1 vs CA3	11.43	1.937	No	ns	-14.38 to 37.24
34	CA1 vs CA4	-7.891	1.337	No	ns	-33.70 to 17.92
35	CA2 vs CA3	11.64	1.973	No	ns	-14.16 to 37.45
36	CA2 vs CA4	-7.677	1.301	No	ns	-33.48 to 18.13
37	CA3 vs CA4	-19.32	3.274	No	ns	-45.13 to 6.486
38						

Attachement VI: Print screen of the statistical analysis of Fmax X Dmax – Figure 6B.



1	Table Analyzed	Dmax (mm)				
2						
3	One-way analysis of variance					
4	P value	0.1522				
5	P value summary	ns				
6	Are means signif. different? (P < 0.05)	No				
7	Number of groups	6				
8	F	1.794				
9	R squared	0.2721				
10						
11	Bartlett's test for equal variances					
12	Bartlett's statistic (corrected)	7.635				
13	P value	0.1776				
14	P value summary	ns				
15	Do the variances differ signif. (P < 0.05)	No				
16						
17	ANOVA Table	SS	df	MS		
18	Treatment (between columns)	0.1220	5	0.02440		
19	Residual (within columns)	0.3263	24	0.01360		
20	Total	0.4483	29			
21						
22	Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
23	CTL vs NT	-0.01731	0.3319	No	ns	-0.2454 to 0.2107
24	CTL vs CA1	-0.1090	2.090	No	ns	-0.3370 to 0.1191
25	CTL vs CA2	0.1002	1.921	No	ns	-0.1279 to 0.3282
26	CTL vs CA3	0.04769	0.9145	No	ns	-0.1804 to 0.2757
27	CTL vs CA4	0.003892	0.07080	No	ns	-0.2244 to 0.2317
28	NT vs CA1	-0.09167	1.758	No	ns	-0.3197 to 0.1364
29	NT vs CA2	0.1175	2.253	No	ns	-0.1105 to 0.3455
30	NT vs CA3	0.0650	1.246	No	ns	-0.1630 to 0.2930
31	NT vs CA4	0.02100	0.4027	No	ns	-0.2070 to 0.2490
32	CA1 vs CA2	0.2092	4.011	No	ns	-0.01888 to 0.4372
33	CA1 vs CA3	0.1567	3.004	No	ns	-0.07138 to 0.3847
34	CA1 vs CA4	0.1127	2.160	No	ns	-0.1154 to 0.3407
35	CA2 vs CA3	-0.05250	1.007	No	ns	-0.2805 to 0.1755
36	CA2 vs CA4	-0.09650	1.850	No	ns	-0.3245 to 0.1315
37	CA3 vs CA4	-0.04400	0.8437	No	ns	-0.2720 to 0.1840
38						