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## **Evaluation of the effects of Ankaferd hemostat application on bone regeneration in rats with calvarial defects: histochemical, immunohistochemical and scintigraphic study**

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## **Evaluation of the effects of Ankaferd hemostat application on bone regeneration in rats with calvarial defects**

M. Turgut et al., Evaluation of the effects of Ankaferd hemostat application on bone regeneration in rats with calvarial defects

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### **Abstract**

**Background:** Bone wax, a hemostatic agent, is widely used in craniospinal surgical procedures for a long time, inspite of controversial results regarding its negative influence upon bone regeneration. In this experimental study, the effects of Ankaferd Blood Stopper (ABS), as an alternative hemostatic agent, were evaluated through histochemical, immunohistochemical and scintigraphic studies.

**Materials and methods:** The total of 30 adult female Wistar-Albino rats was randomly divided into three groups: intact control group (n=10), bone wax group (n=10), and ABS group (n=10). Surgically, a 3.0 mm hole in diameter was drilled on the right side of calvarium of the rats using a Class Mini Grinder set in all three groups, as described previously. At the end of 8 weeks, bone healing and connective tissue alterations surrounding drilled calvarial defect areas of the rats were determined via Hematoxylin and Eosin (H&E) and the Mallory's trichrome staining and anti-bone sialoprotein (BSP) immunohistochemistry. Image Pro Express 4.5 program was used for histomorphometric calculation of new bone and fibrotic tissue areas. All statistical analyzes were made with SPSS 25.0 and analysis of variance (one-way ANOVA) followed by Bonferroni post hoc test was performed,  $p < 0.001$  was considered as significance level.

**Results:** Histomorphometrically, it was found that he had the largest hole diameter and the least fibrotic scar area in the bone-wax group. In the bone wax group, it was observed that the material closed the hole and there was only a fibrotic scar tissue in the area between the bone tissue at the edge of the hole and bone wax, and a fibrotic tissue was formed in the bone wax area. During the histological procedure, this bone-wax material was poured and the sections were seen as a gap in this area. In the ABS hemostat group, the smallest hole diameter and the least fibrotic scar tissue were observed. Fibrotic scar tissue close to each other was found in the ABS hemostat and bone wax groups. Histological analysis of samples also showed a statistical significance for fibrotic connective tissue area between groups ( $p < 0.05$ ). Scintigraphically, osteoblastic activity related to blood flow in the animal taken from the group with application of ABS hemostat was more pronounced compared to the other two groups.

**Conclusions:** In our study, it has been concluded that the ABS yields affirmative effects on the bone healing, while bone wax leads to negative impact on the bone regeneration. Scintigraphic, histochemical and immunohistochemical data support the affirmative impact of the ABS hemostat application upon the bone regeneration apart from the quick stop of hemorrhage.

**Key words:** Ankaferd hemostat, bone wax, calvarium, osteogenesis, rat

## INTRODUCTION

Today, cranial defect is one of the most frequently encountered problems in craniofacial reconstructive surgery, but there is still controversy about repair of cranial

defects. Bone wax, which is frequently used as a hemostatic agent in the control of bone bleeding in surgical operations, is the oldest known and cheapest absorbable substance prepared by Horsley in 1892 [1, 14]. It is a mixture of bees wax and isopropyl palmitate and is used in small pieces. Bone wax, which does not have any coagulation mechanism, stops the osseous bleeding in a physical way and it must be removed from the cavity properly after application because it delays bone regeneration [21, 31]. Therefore, it has been suggested that this material should not be used in places exposed to pressure and expected to heal quickly. On the other hand, Ankaferd Blood Stopper (ABS), consisting of *Thymus vulgaris* (thyme), *Glycyrrhiza glabra* (liquorice), *Vitis vinifera* (grape vine), *Alpinia officinarum* (blue ginger), and *Urtica dioica* (nettle) plant extracts, has been used as an alternative hemostatic agent in folk medicine for many years [5, 6, 12]. Recently, it has been widely used in some clinical trials for various tissue injuries as a topical agent for control of minor or major hemorrhages that occur spontaneously, such as gastrointestinal bleeding, or during or following some surgical interventions, including adenoidectomy and tyroidectomy [5, 7]. Thus, it is well-known that it has a very rapid hemostatic effect in clinical practice, but few studies have assessed the influence of ABS hemostat on connective tissue and bone healing. In a previous experimental study by Bulut et al. (2014), it has been reported that use of ABS as a hemostatic agent caused a negative effect on proliferation, number, viability and morphology of fibroblast cells [4]. In similar, Gul et al. (2020) found that fibrosis was significantly higher in the ABS group in mucosal tissue, suggesting its positive effect on wound healing in rats [13].

Recent studies have revealed that bone sialoprotein (BSP) and osteocalcin (OC) are expressed with the initiation of mineralization with type I, type II and type X collagens in the early period of bone formation [18,29]. In past, some researchers have suggested that bone formation is reduced by impaired mineralization in mice with BSP deficiency [18]. Furthermore, it has also been suggested that osteonectin (ON) and alkaline phosphatase (AP) in cells have important roles in bone healing process. Due to their effects on bone cell differentiation and mineral formation, therefore, ABS hemostat can be used for stimulation of bone regeneration and its effects upon bone cells such as osteoblasts and osteoclasts will be determined, if noncollagenous bone related proteins BSP, OC, ON and osteopontin (OP) are evaluated immunohistochemically as bone formation markers.

On the other hand, three-phase bone scintigraphy is a non-invasive technique that shows the vascularity and osteoblastic activity of the bone. In their study using different bone grafts, Aygit et al. (1999) evaluated vascularity and osseous changes with bone scintigraphy. In this study, they noted that bone scintigraphy could be used as a non-invasive method to

evaluate implant vascularity because of histological changes and scintigraphic findings [2].

Therefore, the objective of this study was to analyze the changes that occur in the bone tissue of rats subjected to bone wax and ABS hemostat applications, which are used as hemostatic agents in various surgical procedures for a long time, comparatively through histochemical, immunohistochemical and scintigraphic studies.

## **MATERIALS AND METHODS**

### **Animals and experimental groups**

In this study, 30 adult 200 gr female Wistar-Albino rats were used for the experiment. The experimental protocol was approved by the Ethical Committee of Aydın Adnan Menderes University (HADYEK 64583101/2016/78). Animals were housed in rat cages in standard conditions ( $24\pm 2^{\circ}\text{C}$  and  $50\pm 5\%$  humidity), exposed to 12:12-h light/dark cycle, fed with standardized rodent chow and tap water ad libitum access. Rats were divided into three groups as intact control group (n=10), bone wax group (n=10), and ABS hemostat group (n=10). Then, critical-sized burr-hole defects (diameter = 3.0 mm) were produced in the calvarium of all rats under general anesthesia. At the end of the 8 weeks all animals were sacrificed following scintigraphical study and cranial tissue samples were taken from the defects for histological histochemical and immunohistochemical analyses.

### **Surgical procedure for burr hole production in rat calvarium**

In the current study, all surgical procedures were performed under general anesthesia provided with intraperitoneal combination of ketamine (50 mg/kg) and xylazine (5 mg/kg) anesthesia. Following heads of rats were shaved in an area of about 2 cm in diameter and cleaned with baticon, a hole 3.0 mm in diameter was opened on the right sides of the rats' calvarium under sterile conditions, using an electric drill (CLASS Mini Grinder sets, PRC), and then the wound was closed, as described previously by Başaloğlu et al. (2021) [3] (Fig. 1).

### **Three-phase bone scintigraphy**

Before sacrifice of the animals for histological examination, one rat was randomly selected from each group for scintigraphic study of the calvarium defect.

Dynamic images were obtained immediately after injection of 37MBq Tc99m methylene diphosphonate (MDP) injection into the tail vein and static images obtained after 2-h of injection with a double-headed Siemens syngo via gamma camera. Static images were acquired with a parallel hole collimator in a 256x256 matrix as 500 Kcount anterior and posterior views. Then, 10-minute images were obtained from the rats' calvarial defect regions with a pin hole collimator and one of them was used as a representative figures of the groups. Tracer uptake was evaluated semiquantitatively in addition to visual evaluation. Region of interest (ROI) was defined around the extracted side and the accumulation of 99mTc-MDP in the regions was measured by software program.

### **Histological examination of rat calvarium**

After sacrifice, the calvarial defects of the rats were taken into 10% formalin solution for 24h before decalcification process using 20% formic acid for 2 months. When softening of the tissue was acceptable tissues were processed with routine histological protocols and embedded in paraffin. 5 µm sections were obtained by Leica RM 2145 microtome and after deparaffinization Hematoxylin and Eosin (H&E), Mallory's trichrome staining and anti-BSP (bs-4729R, Bioss, 1:100) immunohistochemical staining were applied for evaluation of the effects of bone wax on bone osteogenesis in comparison with ABS hemostat. All slides were examined at 10x-20x magnification using an Olympus C5050 camera attached to Olympus BX51 light microscope.

### **Histomorphometry**

For histomorphometric analysis, hole diameter measurements and fibrotic scar areas in the sections were evaluated. Measurement for new ossification areas and fibrotic scar areas was also calculated using Image Pro Express 4.5 (Media Cybernetics, Inc., Rockville, MD, USA) program. Thus, the effects of bone wax upon bone healing were compared with those of ABS hemostat.

### **Statistical analyses**

Data analysis IBM SPSS (Statistical Package for Social Sciences) Statistics for Windows, Version 25.0. (IBM Corp. Released 2017, Armonk, NY). Comparison between groups was made using the one-way analysis of variance method. Normality control was

performed using the Shapiro-Wilk test, one of the obtained error estimates. Since there was no problem of adaptation to normal distribution, the study continued with one-way analysis of variance. Variance homogeneities were examined with Levene test, when there was no homogeneity problem, multi-group comparison was performed with F test, Bonferroni test was used as post hoc tests. In cases where variance homogeneity was not provided, Welch's test was used for multi-group comparison, Dunnett's T3 method was preferred as the post hoc test. All hypothesis checks were carried out at the 0.05 significance level, so  $p < 0.05$  was considered significant.

## **RESULTS**

### **Macroscopic examination**

After the scalp of the rats was skinned, the holes opened in the calvarium were observed macroscopically. When the skull dissection was performed macroscopically, images belonging to all groups were taken. Accordingly, it was determined that the control group was in a normal physiological recovery process. In the bone wax group, it was determined that the material filled the hole and kept the hole closed without any changes during the elapsed time. In the ABS hemostat group, it was observed that the hole opened was closed in appearance and was better than the control group in terms of vascularization (Fig. 2).

### **Microscopic examination**

Orientation of the hole in the bone was adjusted in microscopic sections and serial sections were taken. H-E and Mallory's trichrome staining and anti-BSP immunohistochemical staining were performed to show the general structure in these sections. Importantly, these puncture structures were found to be filled with irregular dense connective tissue called fibrotic in both the control group and the ABS hemostant group (Fig. 2).

In the bone wax group, it was observed that there was fibrotic connective tissue in the area between the bone wax and the bone tissue at the edge of the hole where the material closed the hole, and there was no fibrotic tissue in the bone wax area. During histological follow-up, this bone wax material is seen as a gap in this area in the poured sections (Fig. 3).

When the fibrotic connective tissue structure was examined at higher magnification, it was found that they contained fibroblast-like cells and type I collagen bundles. In the control group, new bone spicules were found, probably formed by intramembranous ossification, where they settled in different regions within the connective tissue. More importantly, it was noted that this connective tissue area was small in the ABS hemostat group and ossification developed in most of the structure. With this appearance, it became the experimental group closest to the normal histological structure of the other groups. Mallory's trichrome staining showed that type I collagen bundles were stained blue, while the newly formed bone areas were red (Fig. 4). When the hole diameters were evaluated statistically, it was seen that the bone wax group had the largest hole diameter. It was found that the smallest hole diameter was in the ABS hemostat group. When the groups were compared with each other, a significant difference was found between control group and ABS hemostat group ( $p < 0.001$ ) as well as bone wax group and ABS hemostat group ( $p < 0.001$ ) (Fig. 5A).

When evaluated in terms of fibrotic scar area, it was found that the lowest scar area was in the bone wax group. We think that the reason for this is that the bone wax material has not been lost yet, which delays wound healing. There is a statistical significance between control group and bone wax group ( $p < 0.001$ ) as well as bone wax group and ABS hemostat group ( $p < 0.001$ ) (Fig. 5B). When evaluated in terms of ossification areas, it was seen that the most ossification area was in the ABS hemostat group, while the least ossified area was in the bone wax group. There is a statistical significance between control group and bone wax group ( $p < 0.001$ ) as well as bone wax group and ABS hemostat group ( $p < 0.001$ ) (Fig. 5C). In terms of vascularization, among the scar areas and ossification areas, it was seen that the most vascular structure was in the ABS hemostat group and the least in the bone wax group. The difference between control group and bone wax group ( $p < 0.001$ ) as well as bone wax group and ABS hemostat group ( $p < 0.001$ ) was found to be significant (Fig. 5D).

### **Bone scintigraphy**

As a result of quantitative examination of the right and left parietal regions of the calvarium of the rats; in the rat in intact control left 10.158 count, right 10.541 count, in bone wax group, left 12.008 count right 12.325 count; and, in the rat in group with application of ABS hemostat, 18.513 count in the left was found as 17.786 count in the right (Fig. 6). In the same rat, however, no significant result was obtained in terms of



vascularity richness in ossification in the right and left parietal regions. Furthermore, a significant amount of adhesion was detected in the rats in ABS hemostat group when compared to that of other two groups. The images were obtained with the pin-hole collimator in each group and they were evaluated visually for vascularity and osteoblastic activity of the region. Based on the results of scintigraphic measurements, osteoblastic activity related to blood flow in the subject taken from the group with application of ABS hemostat was more pronounced compared to that of the other two groups (Fig. 6).

## **DISCUSSION**

The experimental design, in which the rat was used as a model, as in many other experimental studies, due to its advantages in terms of ease of purchase and maintenance, followed the research line established by Başaloğlu et al. [3] Our results clearly showed that osteoblastic activity related to blood flow was evident in the scintigraphy taken from animal with application of ABS hemostat and histological findings confirmed the positive effect of the ABS hemostat application upon the bone healing, while bone wax as a hemostatic agent had a negative impact on the bone regeneration. Also, a statistical difference was found between the groups for the area of fibrotic connective tissue ( $p < 0.05$ ).

The cranium, mostly composed of symmetrical and paired bones, is the most important bone area of the body because it is the main defense system of the brain and brain stem [28]. We can say that it is biologically more inert because it has a different blood supply structure than bone marrow and other bones [22]. It protects the brain from the central nervous system elements that ensure regular and harmonious working coordination of vital functions. Therefore, there are many publications and experimental studies about the healing of cranial defects in the current literature.

When the literature is reviewed on how these substances used in the control of bleeding affect bone healing, it has been determined that significant differences can be detected in first 30 days [1, 9, 15]. In animal experiments, critical dimensional defect was defined as the smallest size bone wound that did not heal spontaneously throughout life without using any material [8, 25]. Such defects tend to fill with fibrous connective tissue and heal rather than bone filling [10, 24]. In their study, Turnbull and Freeman (1974) examined the 2-mm-diameter experimental defects in the parietal bones of rats in terms of healing for 12 weeks and it was observed that there was no significant improvement at the end of this period [30]. Mulliken and Glowacki (1980) examined 2 mm parietal bone defects [19]. While they

could not find a bone filling at the end of the 6th month in control defects, they reported that they encountered bone formation in the experimental group where they applied demineralized bone powder [19].

Howard and Kelly (1969) found that bone wax negatively affected healing in an experimental study they performed in rat tibia [15]. They found that while there was almost complete recovery in the control group of rats that were sacrificed on day 31, the material was not resorbed and delayed osteoblastic activity in the group treated with bone wax [25]. Nooh et al. (2014) used two hemostatic agents, surgical and bone wax, in the bone defects formed in the lateral direction of the right and left metacarpel of goats [21]. Subjects performed sacrificed computed tomography (CT) and histological examinations at 3 and 5 weeks. As a result, they stated that the surgical group was superior to bone wax in terms of bone healing, and bone wax prevented osteogenesis and caused inflammation [21]. Raposo-Amaral et al. performed histological and radiological examination of the use of bone wax in rats with cranial defects [23]. After the surgery, CT scan of the rats was performed at 12th week and as a result, they found that bone wax significantly reduced the bone rate, caused 18% in the healing of the cranial defect and caused fibrosis with infection in rats [23]. Similar results were obtained in animals treated with bone wax in our study, although the drill-opened defect area appeared to be closed macroscopically, this area melted after acid treatment and little fibrotic tissue remained. This situation suggests that bone wax prevents mineralization. In this group, the intensity of osteoclastic activity along with osteoblastic activity is also noteworthy. Bone regeneration and bone resorption continue almost simultaneously. In intact control group, very few new bone spicules were encountered due to intramembranous ossification. The fact that intact control group has a higher ossification pattern compared to bone wax group suggests that it is significant in terms of revealing the negative effect of bone wax on ossification again. This situation is again consistent with similar studies in the literature [11].

On the other hand, however, there are quite a few studies in the literature regarding bone healing, both early and late, and similar to our study, there are also literature studies involving advanced bone healing. In previous histological studies investigating the effects of ABS on the bone tissue, it was found to have an acceleratory effect on the short-term bone healing process in samples taken on the 7th postoperative day [16, 27]. Recently, Kuruoğlu et al. (2017) evaluated the late effects of the combination of ABS and  $\beta$ -tricalcium phosphate (TCP) using immunohistochemical methods and they found the bone healing effects of ABS hemostat in their study [17]. Afterwards, Tanık et al. (2018) also investigated the late period

effects of the combination of ABS and  $\beta$ -TCP on the regeneration of bone tissue in rats [29]. They found that both  $\beta$ -TCP and ABS had positive histological and radiological effects on wound healing and bone formation in samples taken on day 56 after surgery [29]. In the first macroscopic examination we observed in our study, it was determined that the group with the closest appearance of scar tissue to normal structure was group with application of ABS hemostat, suggesting a positive effect of ABS hemostat on wound healing.

Histologically, in the Mallory's trichrome staining, type I collagen bundles were stained in blue, and the newly formed bone areas were red in all groups in our study. In terms of hole diameters, it was determined that the bone wax group had the largest hole diameter and the ABS hemostat group had the smallest hole diameter. A statistically significant difference was found between control group and ABS hemostat group ( $p < 0.001$ ) as well as bone wax group and ABS hemostat group ( $p < 0.001$ ). When evaluated in terms of fibrotic scar area, it was found that the lowest scar area was in the bone wax group. We think that the reason for this is that the bone wax material has not been lost yet, which delays wound healing. There is a statistical significance between control group and bone wax group ( $p < 0.001$ ) as well as bone wax group and ABS hemostat group ( $p < 0.001$ ). In the literature review, however, immunohistochemically studies on bone repair of ABS hemostat are limited. In their experimental study in which they evaluated the use of platelet-rich plasma (PRP) together with autogenous bone graft in the repair of bone defects by immunohistochemical analysis, Nagata et al. (2009) found that the expression of OC and OP was significantly higher in the PRP group [20]. In our study, ossification is present in all three groups, although it seems that the mineralization has not yet been completed. Therefore, absence of any analysis for inorganic material content of bone tissue mineralization and difference of drilled defects from those in humans in terms of bone remodelling are limitations of our study. Moreover, lack of any imaging or mechanical study investigating the bone healing is another limitation of the study. Future studies including immunohistochemical determination of bone related proteins in the early stages of bone healing are needed for better understanding of impact of the ABS hemostat application upon bone remodeling apart from the quick stop of hemorrhage.

It is well-known that three-phase bone scintigraphy is a non-invasive technique showing the vascularity and osteoblastic activity of the bone. Based on their findings in a study, Aygit et al. (1999) stated that bone scintigraphy could be used as a noninvasive method in evaluating implant vascularity because histological changes and scintigraphic findings were parallel [2]. Tc-99m MDP is a radiopharmaceutical that can chemically adhere to all bones in

the skeletal system after intravenous administration. It shows a significant accumulation especially at the points where bone metabolism increases. The common features of radiopharmaceuticals such as MDP, hydroxy diphosphonate (HDP) and hydroxymethylene diphosphonate (HMDP) marked with Tc-99m are that the diphosphonate core in their structure acts like hydroxyapatite, which is the basic building block of bone, and accumulates reversibly on bone surfaces and shows osteoblastic activity depending on blood flow. In this way, all bones belonging to the skeletal system get rid of the soft tissue effect and become visible in a plain state [26]. Diphosphonates attach to hydroxy apatite crystals, one of the main components of the bone matrix, by physicochemical absorption. This amount of adhesion varies according to the blood supply and osteoblastic activity in that area. Organization of hemostasis is primary in fracture or defect repair. When scintigraphic imaging was performed it was not clear between the groups visually. Although MDP is a chemoabsorbable substance in all bones of the skeletal system, it shows a significant accumulation in places where bone metabolism is increased. Radiopharmaceuticals such as MDP, HDP, and HMDP act as hydroxyapatite, which is the main component of bone, and show osteoblastic activity due to a reversible blood flow at the bone surfaces [28]. Although there are not many such studies in the literature, we think that performing bone scintigraphy in weekly periods starting from the defect opening will be more beneficial as it will provide comparable and observable data.

## CONCLUSIONS

Bone fractures due to trauma or surgical operations are conditions that can be encountered in all stages of life. The primary functions of ABS hemostat and bone wax, which are used as hemostatic agents in neurosurgery, orthopedic and traumatology surgery, oral and maxillofacial surgery, are to stop bleeding quickly. Taken as whole, our results showed that:

1. Histological, immunohistochemical and bone scintigraphic data support the positive effects of ABS, consisting of folkloric medicinal plant extracts, on bone healing as well as stopping bleeding rapidly. It was concluded that ABS hemostat also had a positive effect on bone regeneration, while bone wax it had a negative effect on regeneration of the rats' calvarial defects.
2. In various surgical procedures, we think that it is important to choose a blood stopping agent, such as ABS, that will both provide rapid control of bleeding and accelerate regeneration by helping bone repair. However, the effectiveness of this product as a therapeutic modality for possible future clinical applications can interest all types of bones, including extracranial, spongy and cortical, apart from cranial ones.

3. Furthermore, this study also suggests that the anti-inflammatory and antimicrobial properties of the plant ingredients of antihemorrhagic ABS should be evaluated in terms of preventing various complications during the operation and postoperative period in future studies.

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### **Competing interests**

The authors declare that they have no competing interests.

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**Figure 1.** The process of establishing the animal model of bone defect as burr hole on the right side of the rat calvarium using a drill with 3 mm in diameter.

**Figure 2.** Demonstration of the experimentally produced bone lesions in all rat groups. (A) Control group; (B) Bone wax group; and (C) Ankaferd (ABS) hemostat group.

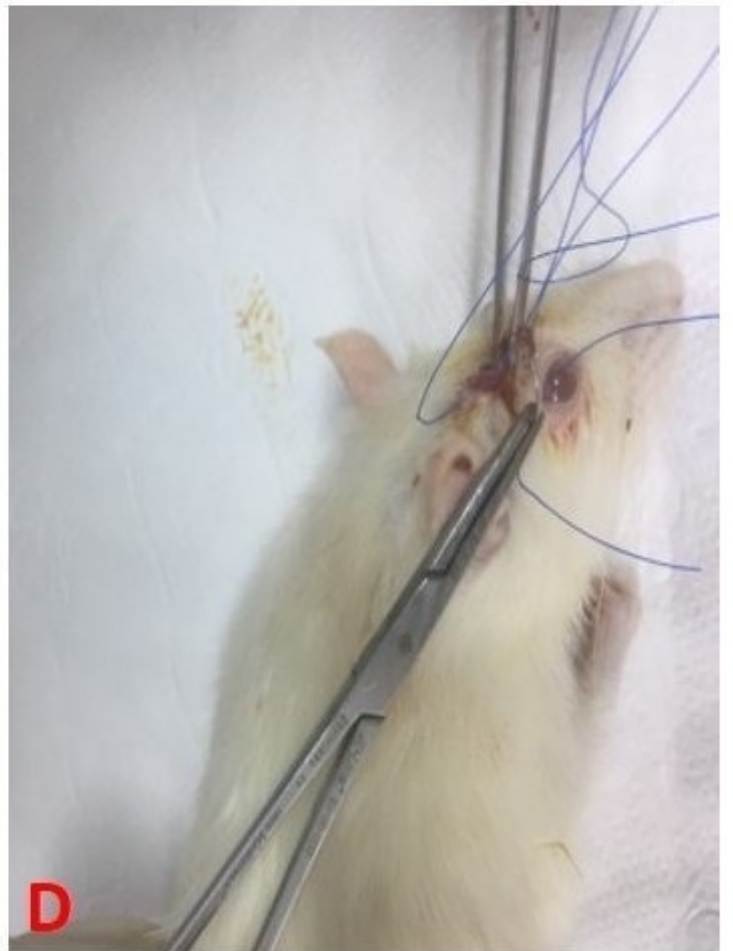
**Figure 3.** Microscopic evaluation of all groups (A) Control group; (B) Bone wax group; and (C) ABS hemostat group. H&E staining.

**Figure 4.** Microscopic evaluation of all groups showing (A) Control group; (B) Bone wax group; and (C) ABS hemostat group. Top row: Mallory's Trichrome staining. Up row: Anti-BSP staining.

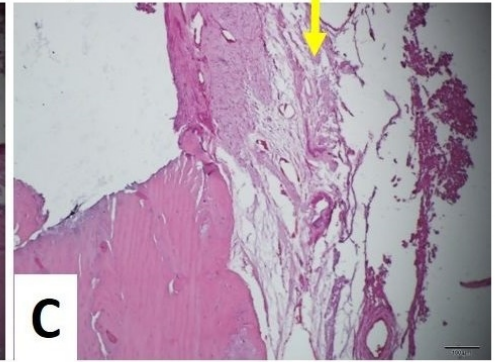
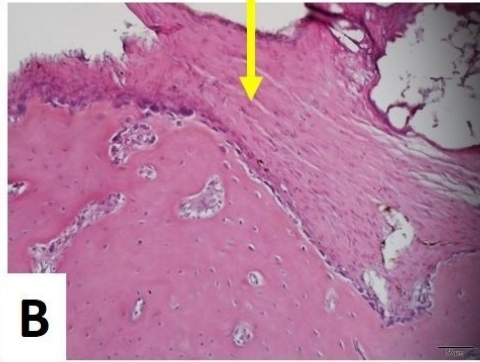
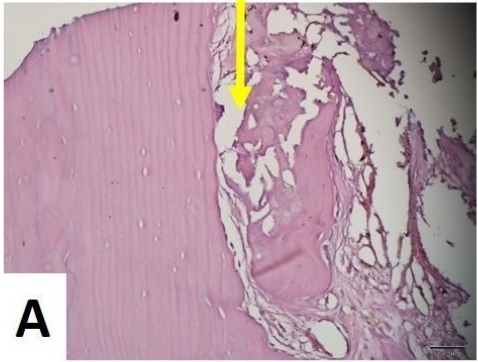
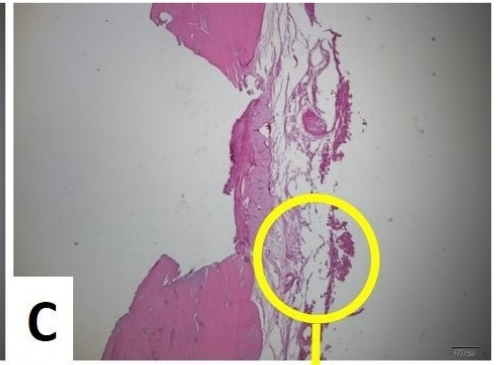
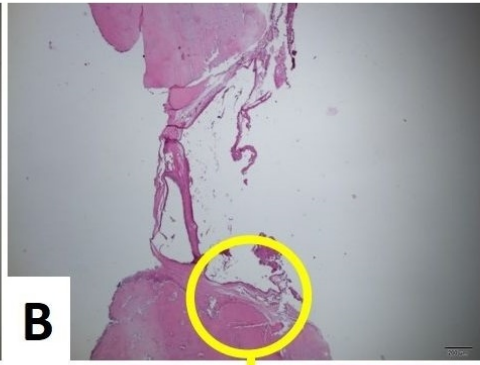
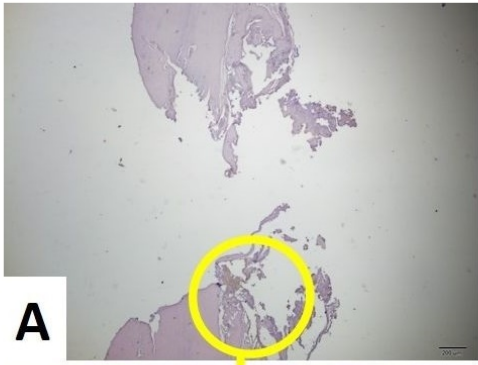
**Figure 5.** Statistical evaluation graph of histomorphometric analysis. Graphical representation of hole diameter (A), fibrotic scar area (B), ossification area (C) and number of vessels (D) in all groups.

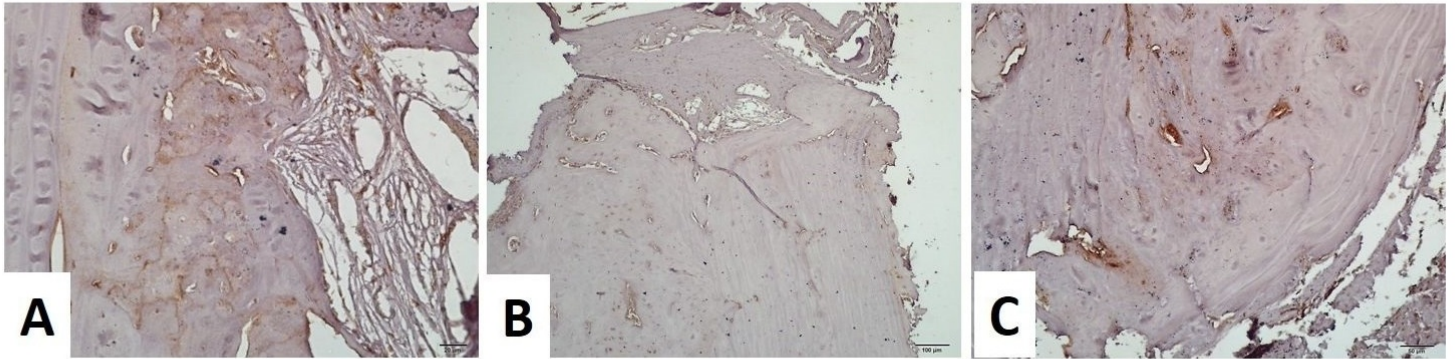
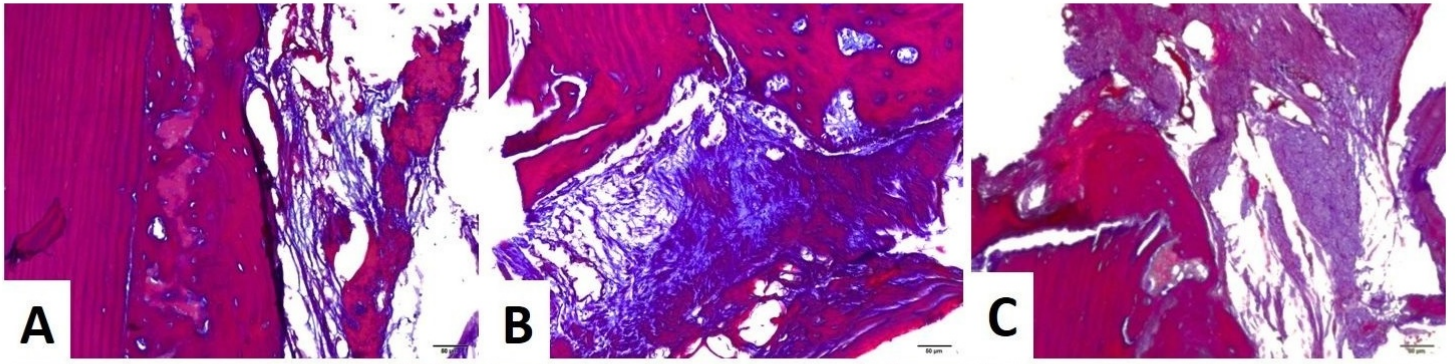
**Figure 6.** Scintigraphic imaging in rats. (A) Control group; (B) Bone wax group; and (C) ABS hemostat group. Results of scintigraphic measurements show more pronounced osteoblastic activity depending on blood flow. In the lower part, the results obtained are also shown graphically.

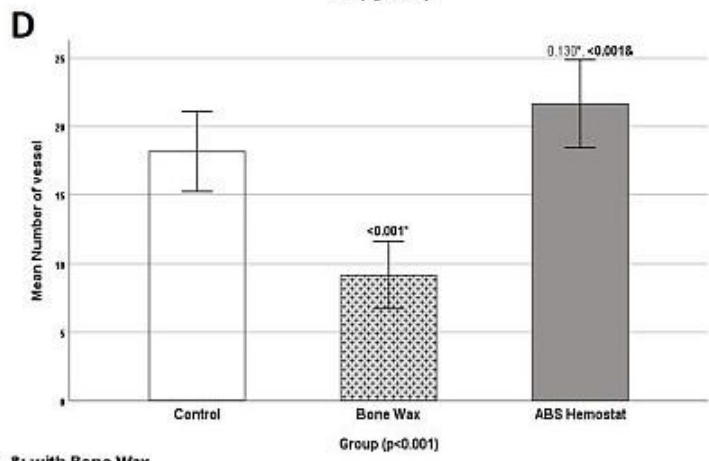
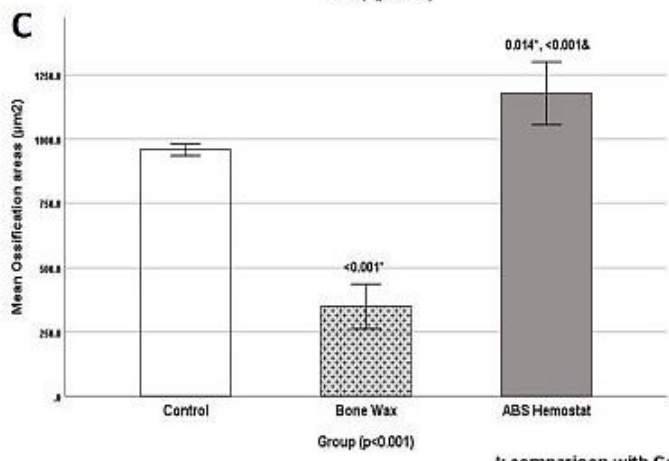
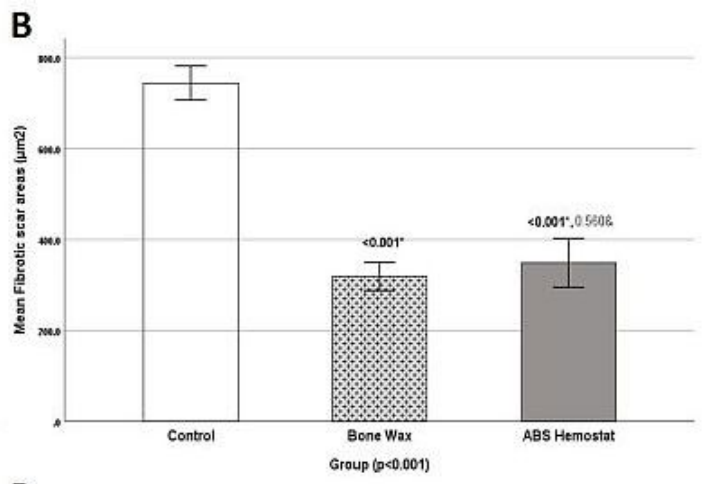
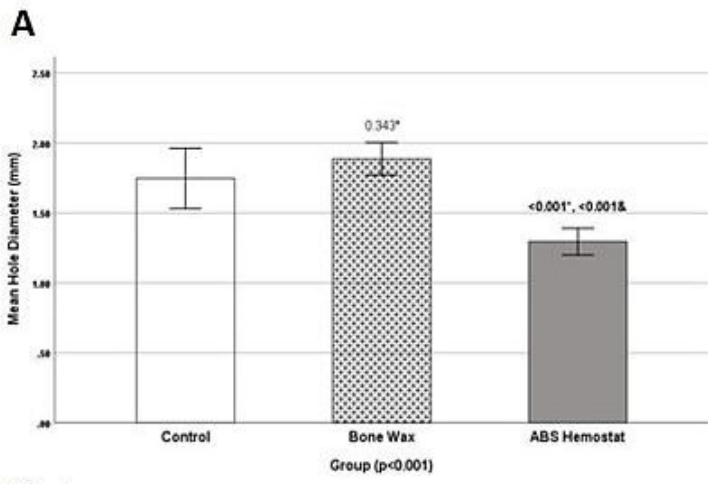












\*: comparison with Control, &: with Bone Wax

**A****B****C**