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Research Article

Effect of direct oral anticoagulant dabigatran on early bone healing: An experimental study in rats

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Abstract

Background. Dabigatran belongs to the new generation of direct oral anticoagulants (DOACs). Its advantages are oral administration and no need for international normalized ratio (INR) monitoring. Although its use has increased, its potential side effects on bone healing and remodeling have not been fully investigated. The present study aimed to evaluate the possible effects of dabigatran on early bone healing.

Methods. Sixteen male Wistar rats were divided into two groups; in group A, 20-mg/kg dabigatran dose was administered orally daily for 15 days, while group B served as a control. Two circular bone defects (d=6 mm) were created on either side of the parietal bones. Two weeks after surgery and euthanasia of the animals, tissue samples (parietal bones that contained the defects) were harvested for histological and histomorphometric analysis. Statistical analysis was performed with a significance level of $\alpha=0.5$.

Results. No statistically significant differences were found between the two groups regarding the regenerated bone (21.9% vs. 16.3%, $P=0.172$) or the percentage of bone bridging (63.3% vs. 53.5%, $P=0.401$).

Conclusion. Dabigatran did not affect bone regeneration, suggesting that it might be a safer drug compared to older anticoagulants known to lead to bone healing delay.

Introduction

The demand for anticoagulant medications has increased as thrombosis remains a major source of morbidity and mortality associated with multiple diseases such as strokes, pulmonary embolism, deep vein thrombosis, etc.[1,2] For many years, anticoagulant drugs included vitamin K antagonists (acenocoumarol, warfarin, etc.) and/or heparins, while better understanding of coagulation cascade events at a molecular level and pharmacokinetics of anticoagulant substances have led to the design of a new generation of anticoagulants.[3] Vitamin K antagonists have recently been replaced with direct oral anticoagulants (DOACs) and, compared to warfarin, have shown the same or greater efficacy and safety.[4] Warfarin administration requires regular monitoring of international normalized ratio (INR) due to its small therapeutic window and significant variability among patients in dosage response.[5,6] DOACs inhibit specific clotting factors, unlike warfarin, which affects several vitamin K-dependent coagulation factors.[7,8] Direct thrombin inhibitors (dabigatran) and factor Xa inhibitors (rivaroxaban, apixaban, and edoxaban) are the most common DOACs.[3] DOACs are delivered orally and do not interfere with the cycle of vitamin K. Therefore, their effect is not affected by diet and does not cause osteopenia or vascular calcification. Dabigatran is a competitive, direct thrombin inhibitor. It inhibits both free and fibrin-bound thrombin, unlike heparin, which only manages to bind to free thrombin.[9] Dabigatran prolongs coagulation markers such as the activated partial thromboplastin time, ecarin clotting time, thrombin time, and dilute thrombin time, but not INR.[10-13] It is administered orally; however, it is not absorbed from the gastrointestinal tract tube in this form but as a prodrug (dabigatran etexilate).[9] Dabigatran etexilate is rapidly absorbed and then converted to dabigatran through hydrolysis in the liver and plasma by esterase as a catalyst.[14] In recent years, the use of DOACs has grown significantly for the prevention of thromboembolic events, and many dental and maxillofacial surgeons now must handle patients who are taking such drugs and require oral surgical interventions.[15] Controversy exists about whether DOACs should be discontinued for 24 hours when teeth extractions or dental implant placement are needed.[16,17] However, a recent systematic review

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and meta-analysis has revealed that if local hemostatic precautions are taken, continuing DOACs therapy does not increase the risk of bleeding in patients having undergone implant surgery.[18] Similarly, Gómez-Moreno et al[19] suggested that patients receiving dabigatran therapy can safely undergo dental implant surgery provided that the last dose is administered 12 hours before and the next one is administered not less than 8 hours after surgery. Based on the literature findings, it seems that until further solid evidence is achieved, before performing dental implant surgery, the physician must weigh the risks and benefits of stopping DOACs while taking into account patient and surgical considerations.[20] Bone regeneration after a trauma is a complex and well-orchestrated sequence of cellular and molecular events that lead to the reconstruction of the damaged area and restoration of its functionality.[21] As a first step, the hemostasis process begins with an accumulation of blood in the fracture area and clot formation. The sequence of events leading to hemostasis includes active coagulation factors, as well as many molecular factors derived from the periosteum, bone marrow, platelets, and surrounding soft tissues.[22] These factors include cytokines, growth factors (vascular endothelial growth factor), the transforming growth factor β1, morphogenetic proteins, and factors related to angiogenesis and osteogenesis. Inside the clot, activated platelets are connected to each other by fibrin fibers, creating a fibrin network, in which various components, such as erythrocytes and leukocytes, are often trapped.[23] This network acts as a scaffold for osteogenesis and is created when the extrinsic coagulation pathway is activated.[23,24] Clotting factors, activated platelets, and other blood cells all play crucial roles in the activation of this pathway.[23,25] Activated platelets keep the clot in place, while polyphosphatases modulate pore size and remodel the fibrillar network. Other cells, such as fibroblasts, leukocytes, and endothelial cells secrete factors that regulate thrombin production. Thrombin is a trypsin-like serine protease that plays a key role in the coagulation cascade.[26] Thrombin leads to the detachment of part of the fibrinogen and turns it into fibrin, while the fibrin polymerization process subsequently begins. Growth factors bind to the fibrin and thus trigger the initiation of bone healing.[23,25] During bone repair, progenitor cells are recruited, and their proliferation and differentiation into osteoblasts and osteoclasts governs subsequent bone formation.[23,25,27-29]

The role of thrombin and its deficiency on bone microstructure and bone density has been investigated in a few studies, and the involved mechanisms have not been fully elucidated. Thrombin has been reported to promote interleukin 6 (IL-6) and prostaglandin E2 (PGE2) expression, favoring osteoclast activation and demineralization of bone matrix by increased expression of RANKL relative to OPG.[30,31] According to Tudpor et al,[32] thrombin receptor impairment causes a drop in the RANKL/OPG ratio, which is linked to a high bone density phenotype. Sivagurunathan et al[33] reported that osteoclast differentiation is inhibited by thrombin, which exerts anabolic effects on osteoblastic lineage cells. Both anticoagulants and antiplatelet drugs can interfere with clot formation, exercising antithrombotic activity with different mechanisms. Their role in bone healing and fracture risk has mainly been investigated with controversial findings.[34-39]

As dabigatran is a direct thrombin inhibitor, it would be interesting to investigate its effects on bone formation and healing after trauma, as there is limited literature showing either potential positive or negative effects on bone formation. Thus, the present study aimed to investigate early bone healing of calvaria bone defects of rats receiving dabigatran and its possible effects on bone regeneration.

Methods

Animal study design

Sixteen male Wistar rats aged 2–3 months with a mean weight of 360.4 g were used in the study. Animal selection, management, and surgery protocol were approved by the Ethics Committee of the Dental School, Aristotle University of Thessaloniki (168139/1229). The animals were fed ad libitum with standard laboratory food pellets during the experiment.

The animals were randomly divided into an experimental (dabigatran) group and a control group (n = 8). In the experimental group, dabigatran (20 mg/kg) was delivered daily for 15 days, starting from 24 hours before the surgical procedure. The dose was chosen after literature research, which revealed many different amounts of doses, with significant variations from 10 mg/kg to 50 mg/kg. An intermediate dose was randomly chosen. Dabigatran capsules were smashed and weighed on a high-accuracy balance to prepare daily doses. As dabigatran is absorbed from the gastrointestinal tract, it was delivered orally. In the control group, no intervention was made.

Surgical procedures

Every animal received antibiotic prophylaxis (Begalin-P PD injection (Sol; Pfizer Hellas, 50 mg/kg subcutaneously) 1 hour before the general anesthesia and after the surgery. The surgical procedure took place 24 hours after the delivery of dabigatran. For general anesthesia, the animals were given ketamine (40–100 mg/kg, i.m., Imalgene, Merial, France, and xylazine 2–5 mg/kg intramuscularly). Before surgery, the dorsal part of the rat calvarium was shaved, and the skin was disinfected using a 10% polyvidone iodine solution. A median sagittal incision was made along the top of the skull, parietal bones were exposed, and two circular calvarial bone defects, 1–2 mm in thickness, were created on both sides of the sagittal suture, with a 6-mm-diameter trephine drill at 1500 rpm under saline irrigation to prevent excessive heating (Figure 1).[40] According to the study of Porto et al[41] in 2012, this size of the defects is critical for the 15-day evaluation

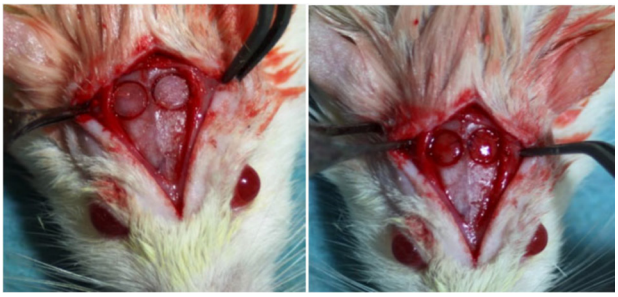

Figure 1. Pictures of the calvaria defects on rats' parietal bones

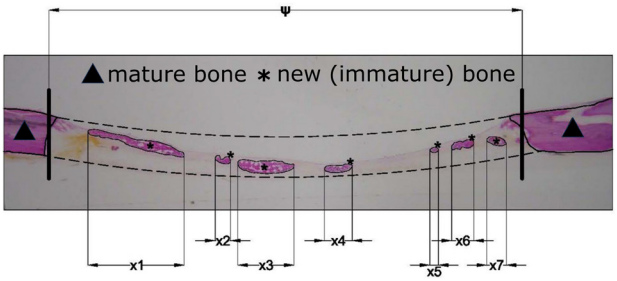

Figure 2. Schematic representation of calculated areas for bone formation on optical microscope images. (ψ: total length of the defect, x1 + x2 + … + xn: length of bridging with newly formed bone)

period. Then, the periosteum and skin were carefully sutured with 4-0 silk (skin) and 4-0 vicryl (periosteum). After the operation, the animals were kept in separate cages following the same diet. There was a complete daily post-surgery follow-up of weight and status.

Fifteen days after the operation, the animals were anesthetized and euthanized by an intravenous administration of pentobarbital (18% solution, 60 mg/kg). Following their euthanasia, whole-body perfusion fixation with 10% neutral buffered formalin was performed, and the cranial bone areas containing the defects were block-sectioned for histological preparation and histomorphometry analysis.

Histological preparation

All the specimens were initially immersed in a 10% formaldehyde solution for fixation, followed by dehydration by sequential immersion of ascending concentrations of alcohol. Subsequently, the specimens were infiltrated by methylmethacrylate by immersion in alcoholic solutions of increasing concentrations of methylmethacrylate. Next, 80-μm methylmethacrylate-embedded tissue sections were prepared for histological evaluation using the EXAKT system (Advanced Technologies GmbH, Norderstedt, Germany). The specimens were cut vertically, and histological sections were duly oriented to coincide with the direction of the defect diameter. The sections were then conventionally stained with toluidine blue/basic fuchsin.

Histomorphometry

The samples were viewed under an optical microscope (Zeiss Axio Lab, Germany), and the development and degree of the new bone maturation were recorded. Digital images were captured (SONY DSC F707) to perform histomorphometry measurements using the special software Image Pro Plus (Media Cybernetics Inc., Rockville, MD, United States). With the above program, it was calculated (Figure 2):

A. The percentage (%) of bone defect regeneration: the surface of newly formed bone/total surface of the defect × 100%

B. The percentage (%) of defect bridging: the length of bridging with newly formed bone/ total length of the defect × 100%

Sample size calculation

Based on our pilot study,[40] we considered that the primary outcome (defect regeneration percentage) in the control group would be approximately 20 ± 10%. Based on this, to detect a difference of ± 15% between the groups, at least seven animals would be needed in each group to reach a power (1-β) of 0.80, with α = 0.05. G*Power v.3.1.9.2 (Frantz Faul, Univerisität Kiel, Germany) was used to calculate the sample size.

Statistical analysis

Statistical analyses were performed using SPSS 20.0 (SPSS, Inc., Chicago, IL, USA). The average percentages of bone regeneration and defect bridging were initially calculated from the two defects for each animal. The histomorphometric parameters were presented as mean ± standard deviation within the groups. The significance of differences between the groups was determined by the t-test for independent samples since the data met the criteria for normal distribution, as indicated by the Shapiro-Wilk test. Statistical significance was determined at $P < 0.05$.

Results

Surgical procedures were completed without complications, and all the animals recovered well from the sedation and interventions. The postoperative period was uneventful, and all the animals completed the study without any bleeding complications, surgical wound dehiscence, signs of infection, or other complications. Macroscopic postmortem inspection showed that none of the defects was completely regenerated. In both groups, histological examination showed partial coverage of defects with newly formed bone, mostly woven (Figure 3). A complete bridging of the defect was noticed in five histological specimens of the dabigatran group (Figure 3A) and in seven histological specimens of the control group (Figure 3C), possibly caused by the remaining periosteum in the wound area. In the rest of the specimens, either the bridging was not complete (Figure 3B), or small islets of newly formed bone existed in the center of the defect, indicating osteogenesis coming from the periosteum and the dura mater (Figure 3D). Between the two groups, there was no statistically significant difference either in

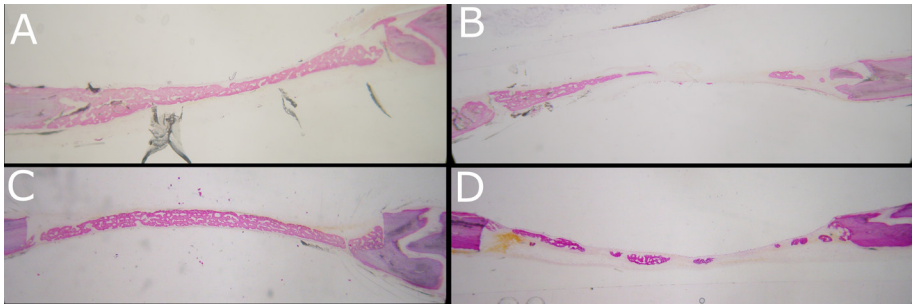

Figure 3. Representative images of bone regeneration. (A) Complete bridging (dabigatran group), (B) Partial bridging (dabigatran group), (C) Complete bridging (control group), (D) Islets of woven bone (control group)

the percentage of newly formed bone or in the percentage of defect bridging (Table 1).

Table 1. Mean percentages (%) of bone regeneration and defect bridging between groups

	Dabigatran group	Control group	*P* value
Bone regeneration (%)	21.9 ± 6.0	16.3 ± 11.2	0.172
Defect bridging (%)	63.3 ± 14.5	53.5 ± 22.3	0.401

Discussion

To the best of our knowledge, the present study is the first to investigate the role of dabigatran in early bone healing in rat calvarial bone defects. The results showed that systematic delivery of dabigatran did not affect bone regeneration, consistent with a similar study by Kerimoglu et al,[11] in which the authors examined the effect of dabigatran in tibial fractures in rats. In their study, four groups received different doses of dabigatran with various delivery durations. (Group 1: 10 mg/kg for 14 days, group 2: 10 mg/kg for 28 days, group 3: 50 mg/kg for 14 days, group 4: 50 mg/kg for 28 days). Their study showed no statistically significant difference between the groups receiving dabigatran for 14 (groups 1 and 3) or 28 days (groups 2 and 4) regarding the radiologic or histomorphometric evaluations. Therefore, the drug dose seems not to affect the outcomes. However, there was a statistically significant difference when the groups were compared regarding the duration of drug delivery (comparison between groups 1-2 and 3-4), which underlines the possible effect of the delivery duration.

Moreover, Fusaro et al[10] compared the effect of dabigatran related to warfarin administration on bone structure and vascular calcification in rats. The animals were divided into three groups: the first as normal control (untreated), the second with delivery of dabigatran (1 mg/g of food, 15-30 g a day in total), and the third with delivery of warfarin in a dose to reach a concentration sufficient to obtain an INR between 2 and 3. After sacrificing the animals, the femur, tibia, and vertebrae were collected and stored in ethanol for immunohistochemical and morphometric analyses of bone remodeling. In the warfarin group, a histomorphometric study of the femur and vertebrae revealed dramatically reduced bone volume and increased trabecular separation. Vertebral examination revealed that the rats receiving dabigatran had more trabecular tissue. Except for maximum erosion depth, which was higher in warfarin-treated rats, possibly indicating increased osteoclastic activity, osteoblast activity and resorption parameters were comparable between the groups. As a result, warfarin was linked to increased bone formation and activation frequency, possibly leading to increased bone remodeling with higher osteoclast activity. Rats treated with warfarin had lower bone volume, greater trabecular separation, and higher turnover than those treated with dabigatran or the control group. These findings imply that compared to warfarin, dabigatran has a higher bone safety profile. These variations may translate into a decreased incidence of fractures in dabigatran-treated individuals since warfarin medication impacts bone by diminishing trabecular size and structure, increasing turnover, and reducing mineralization. Similar to dabigatran, the production of massive calluses and an increase in bone mineral density reported in a rat model of femur fracture suggest that rivaroxaban (factor Xa inhibitor) may beneficially affect fracture healing.[42]

Brent et al[43] used male and female C57BL/6 mice and evaluated the role of dabigatran mixed in chow in bone mineral density and bone mineral content (BMC) of various murine bones. They concluded that despite the relatively large dose of dabigatran utilized (1.52 and 1.70/g body weight for females and males, respectively), neither male nor female mice exhibited any significant detrimental effects on bone tissue, apart from a small favorable site-specific effect at the tibial cortical bone in female mice.

Numerous studies have tried to elaborate on the effect of DOACs on increased fracture risk or new-onset osteoporosis. However, although they have been found superior compared to vitamin K antagonist (VKA) anticoagulants,[42,44-46] there are no clear findings on whether differences exist between the different classes of DOACs[47-50] and differences exist between studies. In a recent network meta-analysis with osteoporotic fractures, including 321 844 patients with a follow-up of two years, it was found that from all the DOACs currently on the market, apixaban has the lowest likelihood of developing an osteoporotic fracture.[50] Another population-based

cohort study[48] found that when opposed to taking warfarin, patients with atrial fibrillation who utilize DOACs may experience a lower risk of osteoporotic fracture; however, the kind of DOAC does not appear to change the fracture risk.

Concerning other new generations of anticoagulants, a few studies have evaluated their effect on bone. Xia et al[51] compared the effect of heparin with rivaroxaban on rats and examined the levels of calcium and phosphorus in serum, markers of bone formation (e.g., alkaline phosphatase and PINP), and markers of bone resorption (pyridinoline and deoxypyridinoline) for assessing bone metabolism. Additionally, energy x-ray absorptiometry and a CT scan were used to compare trabecular and cortical bone microstructures. The serum calcium and phosphorus levels were comparable between the heparin and rivaroxaban groups, but the markers of bone formation and bone resorption differed. The group that received heparin showed higher bone resorption markers but lower activity and levels of bone formation markers. Rivaroxaban, on the other hand, only caused PINP levels to drop. Heparin hindered bone growth and accelerated bone resorption, according to the study's findings. Both trabecular and cortical bone morphometric parameters were impacted by heparin. Cortical volume was decreased in rats receiving heparin treatment, according to micro-CT studies of cortical bones. However, following rivaroxaban therapy, no appreciable change was observed. The researchers concluded that rivaroxaban had less detrimental effects on bone microstructure than heparin. Klüter et al[52] evaluated the effect of administrating rivaroxaban at a dose of 3 mg/kg body weight per day for 28 and 49 days after creating femur fractures on Wistar rats. They concluded that rivaroxaban did not impair fracture healing, although they emphasized the small number of animals in their study.

Significant findings can be achieved from in vitro studies employing DOACs and other anticoagulants in different bone cells, although here also, the results are controversial. Rocha et al[53] evaluated the effect of dabigatran (Pradaxa® capsule, Boehringer, Ingelheim am Rhein, Germany) on different cell cultures, including osteoclasts. Although heparin effects have been documented on osteoclasts,[54] data on DOAC effects are not yet available. They used bone marrow-derived osteoclasts isolated from the femurs and tibiae of C57BL/6 mice, osteoblasts derived from calvaria fragments of newborn Wistar Hannover rats, and a pre-osteoblastic cell line (American Type Culture Collection, Manassas, VA, USA). They reported reduced osteoclast differentiation at the highest tested concentrations (2 µg/mL and 3 µg/mL), as verified by TRAP staining and downregulation of CTSK expression, which is a key marker of osteoclast differentiation and activity. They also reported reduced osteoblast differentiation, as confirmed by reduced alkaline phosphatase activity and mineralized matrix formation. They also administered 428.5 µL of an aqueous solution of dabigatran etexilate at 100 µg/mL concentration twice daily in 5 rats for 28 days and investigated whether their BMCs retained their ability to differentiate into osteoclasts. They found that despite no significant differences in the TRAP-stained osteoclasts, their resorption capacity was significantly restrained.

Opposite results were found in an in vitro study by Winkler et al.[27] The study aimed to investigate the effect of melagatran, a direct thrombin inhibitor, on human osteoblasts. Osteoblast cultures were derived from cancellous bone from 6 individuals, harvested during total knee replacement. Melagatran, dalteparin, and unfractionated heparin (UFH) were added to primary osteoblast cultures. Cell number, protein synthesis, mitochondrial and alkaline phosphatase activity, and collagen type I synthesis were evaluated. In the highest investigated concentration, melagatran only reduced the cell count to 84% of the control group after 15 days of incubation. In contrast, the reduction of cell count was far more pronounced under the influence of dalteparin (39%) and UFH (10%). Melagatran showed less inhibitory in vitro effects on human osteoblasts than dalteparin or UFH.

In vitro effects of other DOACs have shown that rivaroxaban can inhibit the proliferation of female-derived osteoblasts. Gigi et al[55] investigated the direct effects of rivaroxaban on bone biology; the in vitro model demonstrated that osteoblastic mineralization was unaffected. The study's findings indicated that rivaroxaban inhibits the first stage of bone formation but does not affect later stages (i.e., bone mineralization).

The present study had some limitations. For example, the evaluation of bone healing at different time intervals was not conducted, nor was the administration of different doses of dabigatran for different perioperative periods. However, since dabigatran affects the coagulation cascade, it may be hypothesized that any effect would occur in the early stages of the defect healing. Moreover, our study would have benefited from using micro-computed tomography to obtain 3-D quantitative data of the defect regeneration.

Conclusion

Under the limitations of the present study, systematic delivery of dabigatran seems not to affect bone regeneration in calvarial defects in rats. There were no statistically significant differences between the two groups, the control and the one with dabigatran administration, either in the percentage of newly formed bone or in the percentage of defect bridging. However, the findings were slightly better for the dabigatran group. This finding adds another benefit to using the new generation of coagulants. However, further studies are needed, both in vitro and in vivo, to elucidate the underlying mechanisms of bone healing in patients receiving DOACs, as from the limited literature, it seems that gender, duration,

and administration dose may be factors that can play a significant role. Further in vivo studies should involve multiple DOACs to clarify which factors may contribute to the different results obtained with the various types of drastic DOAC substances.

Authors' Contribution

Conceptualization: Ioanna Kyriakaki, Nikolaos Dabarakis.
Formal analysis: Ioanna Kyriakaki, Theodora Karanikola, Theodoros Lillis, Eleana Kontonasaki.
Investigation: Ioanna Kyriakaki, Theodoros Lillis, Theodora Karanikola.
Methodology: Ioanna Kyriakaki, Theodoros Lillis, Theodora Karanikola.
Project administration: Nikolaos Dabarakis.
Supervision: Nikolaos Dabarakis.
Writing–original draft: Theodora Karanikola, Eleana Kontonasaki, Nikolaos Dabarakis.
Writing–review & editing: Theodoros Lillis, Eleana Kontonasaki, Nikolaos Dabarakis.

Competing Interests

The authors declare that they have no financial and non-financial competing interests concerning the publication of their work during submission.

Data Availability Statement

The data produced during the present study are available from the corresponding author upon reasonable request.

Ethical Approval

The present study was approved by the Protocol Approval Committee of the Directorate of Veterinary Medicine of the Region of Central Macedonia for implementation of rules and ethics during experimentation on laboratory animals in accordance with Presidential Decree 56/2013, at its meeting on June 30, 2017, with the protocol number 168139/1229.

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