Thrombosis Research 186 (2020) 45–53

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Full Length Article

Inhibitory effects of dabigatran etexilate, a direct thrombin inhibitor, on osteoclasts and osteoblasts

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ARTICLE INFO

Keywords:
Anticoagulants
Dabigatran
Thrombin
Osteoclasts
Osteoblasts
Cell Culture Techniques

ABSTRACT

Introduction: Anticoagulants are widely used in orthopedic surgery to decrease the risk of deep vein thrombosis. While significant bone impairment is induced by long-term heparin therapy, little is known about the effects of direct oral anticoagulants (DOACs). Herein, we investigated the effects of dabigatran etexilate (Pradaxa®), a DOAC inhibitor of thrombin, on bone cells using *in vitro* and *ex vivo* cell culture models.

Materials and methods: Osteoblasts and osteoclasts exposed to different concentrations of dabigatran etexilate and untreated cells were assayed for cell differentiation and activity. Favorable osteogenic conditions for osteoblasts were tested using titanium with nanotopography (Ti-Nano). In addition, mice treated with a dabigatran etexilate solution had bone marrow cells analyzed for the ability to generate osteoclasts.

Results: Dabigatran etexilate at concentrations of 1 μg/mL and 2 μg/mL did not impact osteoclast or osteoblast viability. The drug inhibited osteoclast differentiation and activity as observed by the reduction of TRAP + cells, resorption pits and gene and protein expression of cathepsin K. Consistently, osteoclasts from mice treated with dabigatran showed decreased area, resorptive activity, as well as gene and protein expression of cathepsin K. In osteoblast cultures, grown both on polystyrene and Ti-Nano, dabigatran etexilate reduced alkaline phosphatase (ALP) activity, matrix mineralization, gene expression of ALP and osteocalcin.

Conclusions: Dabigatran etexilate inhibited osteoclast differentiation in *ex vivo* and *in vitro* models in a dose-dependent manner. Moreover, the drug reduced osteoblast activity even under optimal osteogenic conditions. This study provides new evidence regarding the negative overall impact of DOACs on bone cells.

1. Introduction

Long-term oral anticoagulation is indicated for the prevention and treatment of thromboembolic diseases. Coumarins or vitamin K antagonists have been the first choice therapy for over 50 years [1]. Nevertheless, over the last few years, direct oral anticoagulants (DOACs) have been approved, with the advantages of having more predictable pharmacokinetics and fewer drug interactions [2]. These

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https://doi.org/10.1016/j.thromres.2019.12.014
Received 23 October 2019; Accepted 19 December 2019
Available online 23 December 2019

drugs avoid the drawbacks associated with warfarin and the need of dosage adjustments and monitoring [3]. Dabigatran is a direct thrombin inhibitor (DTI) of the DOAC class that binds directly to thrombin and blocks its interaction with its substrates [4]. Commercially, the drug is available in the mesylate salt form, Pradaxa® (dabigatran etexilate mesylate). After oral administration, the prodrug, dabigatran etexilate, is converted by esterases to its active form, dabigatran – a potent, competitive and reversible direct inhibitor of the active site of thrombin [5]. Dabigatran was approved as being effective in the prevention and treatment of venous thromboembolism, stroke and systemic embolism in individuals with nonvalvular atrial fibrillation [6].

DOACs are a novelty on the market and the knowledge of possible risks of their use for treatment is essential in order to improve clinical practice by making them more effective and safer. In this regard, studies investigating the potential side effects of anticoagulants on bone remodeling have been reported elsewhere [7–9]. Bone remodeling is a complex and continuous process maintained as a tightly coupled balance between bone deposition by osteoblasts and bone resorption by osteoclasts [7]. Abnormalities in this mechanism may lead to imbalance in bone homeostasis, triggering skeletal disorders [9]. Osteopenia and osteoporosis are acknowledged side effects of heparins after long-term treatment [1,10,11]. Warfarin could have direct negative effects on bone by the inhibition of γ-carboxylation of osteocalcin as well as indirect effects, because individuals treated with warfarin may limit their dietary intake of foods rich in vitamin K [12]. In contrast, the effects of DOACs on bone cells are poorly studied. DTIs and factor Xa have been evaluated *in vitro* and *in vivo* and, although no consensus exists, some results have suggested their negative impact on bone cells and structure [11,13–17].

Since the publication of the RE-MODEL clinical trial [18], the use of dabigatran has increased for the prevention of venous thromboembolism in individuals submitted to elective hip or knee arthroplasty. These are clinical situations in which a well-preserved bone cell function is required for successful interaction between the cells with the prosthesis interface and hard tissue formation. Therefore, the purpose of the present study was to investigate the effects of dabigatran etexilate (Pradaxa®) on osteoblasts and osteoclasts and the interaction of osteoblasts with titanium (Ti) surfaces.

2. Materials and methods

2.1. Preparation of the dabigatran etexilate (Pradaxa®) solution

A stock solution of dabigatran etexilate (100 μg/mL) was prepared by the accurate weighing of 142.73 mg of the contents of the Pradaxa® capsule (Boehringer, Ingelheim am Rhein, Germany) corresponding to 50 mg of dabigatran etexilate. This amount was diluted in 500 mL of ultrapure water and sonicated for 10 min. Prior to the experiments, the dabigatran etexilate stock solution (100 μg/mL) was filtered through a 0.22 μm membrane filter [33 mm in diameter, PVDF, Millipore, Burlington, MA, USA]. Filters were pretreated in order to eliminate the binding of dabigatran etexilate to the membrane filter. The devices were soaked in a passivating solution (Tween™ 20, 5% *w/v*, Croda Health Care, East Riding, UK) maintained overnight at room temperature and washed with distilled water prior to use. The filtered solutions were stored at 2–8 °C protected from light until analysis. The stability of the solutions was evaluated for 30 days by high-performance liquid chromatography (HPLC). The working solutions (1–6 μg/mL) were prepared by the proper dilution of the stock solution (100 μg/mL) in alpha minimum essential medium (α-MEM, Invitrogen Life Technologies, Grand Island, NY, USA). The detection of dabigatran etexilate after dilution in the culture medium was also evaluated by HPLC analysis.

HPLC analysis was performed by adapting the method described by Bernardi et al. [19]. A Shimadzu HPLC system (Kyoto, Japan) consisting of a quaternary pump, an autosampler, and a diode array detector (DAD) was used. An Agilent C18 column (250 mm, 4.6 mm, 5 μm particle size) was also used. Ultraviolet (UV) detection was performed at 225 nm. The mobile phase was an acetonitrile: triethylamine solution, pH 6.0, adjusted with phosphoric acid (65:35 v/v). The optimized flow rate was 1.0 mL/min, and a 50 μL aliquot of the sample was injected during each run.

To assess the effects of the active principle, cells were treated with dabigatran (Dabigatran-D3 100 μg/mL solution, acetonitrile with 10% 0.01 N HCl, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) at concentrations of 0.1 μg/mL, 0.3 μg/mL, 3 μg/mL, and 6 μg/mL. Untreated cells under the same conditions were used as controls for the *in vitro* experiments.

2.2. Animals

Male C57BL/6 mice (six weeks of age) and Wistar Hannover rats (three days of age) were acquired from the center of animal care of the Federal University of Minas Gerais (UFMG) and University of São Paulo (USP). Animals were treated in conformity with the regulations of the Institutional Ethics Committee of the universities (No. 247/2018 and No. 2018.1.562.58.0).

2.3. Bone marrow cell-derived osteoclasts

Bone marrow cells (BMCs) were isolated from the femurs and tibiae of C57BL/6 mice and cultured with α-MEM (Thermo Fisher Scientific, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco®, CA, USA). Cells were grown in a humidified atmosphere containing 5% CO_2 at 37 °C and a soluble macrophage colony-stimulating factor (M-CSF, 30 ng/mL, R&D Systems, MN, USA) for three days. The adherent cells (osteoclast precursors) were then plated in 96-well microplates (Corning Inc., Corning, NY, USA) at a density of 2×10^4 cells/well, and cultured in α-MEM (Thermo Fisher Scientific) containing MCSF (30 ng/mL, R&D Systems) and receptor activator of nuclear factor kappa-B ligand (RANKL 10 ng/mL, R&D Systems).

2.4. Tartrate-resistant acid phosphatase (TRAP) staining

The BMC-derived osteoclasts were fixed after five days of culture with acetone, citrate, and 37% formaldehyde and stained with a TRAP commercial kit (Sigma-Aldrich) according to the manufacturer's instructions. The experiments were carried out in triplicate and performed at least twice. Multinucleated (three or more nuclei) TRAP+ cells were considered to be osteoclasts. The cells were counted and measured in mm. The images were captured using ImageJ software (National Institutes of Health, Betesda, MD, USA) and the Cytation™ 5 Cell Imaging Multi-Mode Reader (BioTek, Winooski, Vermont, USA).

2.5. Resorption pit assay

Osteoclasts were generated and cultured in 96-well osteoassay microplates (Corning Inc.). Resorption and pit formation area were measured after 10 days of osteoclast differentiation. The experiments were performed in triplicate. The pits were observed and captured under a microscope at 4× magnification. The results are reported as percent pit areas/well using the contouring tools of Leica Application Suite software (Leica Microsystems, Hessen, Wetzlar, Germany).

2.6. Calvaria-derived osteoblasts

Osteoblasts were harvested from calvaria fragments of eight newborn Wistar Hannover rats by sequential enzymatic digestion. Briefly, a 0.25% trypsin solution and 0.2% type II collagenase (all from Gibco®) were added to the fragments to isolate the cells [20]. Cells were counted and plated in 24-well culture microplates (Corning Inc.) at a

cell density of 2×10^4 cells/well. The osteoblasts were cultured in α-MEM osteogenic growth medium (Invitrogen Life Technologies) supplemented with 10% FBS (Gibco®), 100 μg/mL gentamicin (Gibco®), 5 μg/mL ascorbic acid (Gibco®), and 7 mM β-glycerophosphate (Sigma-Aldrich) for up to 14 days. During the culture period, the cells were kept at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The medium was changed every three days.

2.7. Osteoblast cell line

The MC3T3-E1 pre-osteoblastic cell line (American Type Culture Collection, Manassas, VA, USA) was also used for the experiments. The cells were plated in 24-well polystyrene microplates (Corning Inc.) at a density of 2×10^4 cells/well and cultured for up to 14 days. The medium for MC3T3-E1 cells was α-MEM (Invitrogen Life Technologies) supplemented with 10% FBS (Gibco®), 100 U/mL penicillin (Invitrogen Life Technologies), 100 μg/mL streptomycin (Gibco®), 5 μg/mL ascorbic acid (Gibco®), and 7 mM β-glycerophosphate (Sigma-Aldrich). During the culture period, the cells were kept at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air, and the medium was replaced every three days.

2.8. Alkaline phosphatase (ALP) activity

After seven days of osteoblast culture *in situ*, ALP activity was qualitatively evaluated by Fast red staining. This staining results in an insoluble Naphthol-Fast Red complex that precipitates where cells show ALP activity. The medium was removed and the cultures were incubated with 1 mL/well of a mixture of 0.9 mM naphthol AS-MX phosphate (Sigma-Aldrich) and 1.8 mM Fast red TR Salt (Sigma-Aldrich). Before mixing, naphthol AS-MX phosphate was solubilized with 4 mg/mL dimethylformamide (Merck KGaA). The plates were kept at 37 °C for 30 min and the solution was removed and dried overnight. The experiments were undertaken in quadruplicate. *In situ* ALP was quantified by counting pixels with Leica Application Suite software (Leica Microsystems). The results are reported as percent stained areas in relation to the control group.

2.9. Alizarin red staining

After 14 days of osteoblast culture, extracellular matrix mineralization was quantified using alizarin red staining. The culture medium was removed, and the wells were fixed in 10% formalin for 2 h at room temperature, dehydrated and stained with 2% alizarin red (Sigma-Aldrich), pH 4.2, for 10 min. For quantitative analysis, calcium content was determined by a colorimetric method. Briefly, 280 mL of 10% acetic acid were added to each well and the plate was incubated for 30 min under shaking at room temperature. This solution was vortexed for 1 min, heated to 85 °C for 10 min, and transferred to ice for 5 min. The samples were then centrifuged, and 100 mL of the supernatant was mixed with 40 mL of 10% ammonium hydroxide. The optical density values were evaluated spectrophotometrically with the mQuant® plate reader (BioTek) at 405 nm. The experiments were carried out in quadruplicate.

2.10. Ti surfaces

Machined discs of commercially pure grade two Ti 13 mm in diameter and 2 mm thick (Realum, São Paulo, SP, Brazil) were polished with 180, 320 and 600 grit silicon carbides, cleaned by sonication, and washed with toluene. The discs were conditioned with a solution of 10 N H_2SO_4 and 30% aqueous H_2O_2 (1:1 v/v) for 4 h at room temperature under continuous agitation in order to obtain the nanotopography surface. Non-conditioned discs were used as control (machined). All discs were autoclaved before the cell culture experiments.

2.11. Mice treated with dabigatran etexilate solution and ex vivo model

Ten male C57BL/6 mice (six weeks) were randomly divided into two groups: dabigatran etexilate treatment ($n = 5$) and untreated controls (n = 5). The standardized dose of treatment was calculated proportionally to that recommended for humans, *i.e.*, one Pradaxa® capsule (150 mg) every 12 h [18]. Considering the recommended daily dose of 300 mg/day for individuals weighing 70 kg and the mean animal weight of 20 g, a dose of 85.7 μg/day was used in this study. The dose corresponds to 428.5 μL of the aqueous solution of dabigatran etexilate at 100 μg/mL concentration, administered twice daily. The treatment was administered by gavage under inhalatory sedation with 2% isoflurane for 28 days. The animals were housed five to a cage with free access to food and water. At the end of the study, the animals were anesthetized and euthanized. BMCs were isolated from femurs and tibiae and cultured as described above.

2.12. Cell viability assay

Cell viability/proliferation of dabigatran etexilate-treated cultures of osteoclasts and osteoblasts were evaluated by measuring the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT, Sigma-Aldrich) to purple formazan crystals, according to manufacturer instructions. Briefly, 20 μL of MTT solution in 96-well and 1 mL in 24-well culture microplates (Corning Inc.), 5 mg/mL in PBS was added to each well, and the plates were incubated for 3 h at 37 °C. The medium was removed and the MTT crystals were solubilized with 200 μL dimethyl sulfoxide (DMSO, Sigma-Aldrich) or isopropanol acid solution (100 mL isopropanol and 134 μL HCl) at room temperature. The spectrophotometric absorbance of each sample was then measured at 540 nm (DMSO dilution) and 570 nm (isopropanol dilution). The experiments were carried out in quadruplicate and the results are reported as percent optical density of viable cells in relation to the control group.

2.13. Real-time polymerase chain reaction (RT-qPCR)

Gene expression was evaluated in osteoclasts and osteoblasts and compared to that of untreated cells. After three and seven days of culture, total ribonucleic acid (RNA) was extracted with TRIzol® reagent (Invitrogen Life Technologies) followed by the SV total RNA isolation system (Promega, Madison, WI, USA), according to the manufacturer's instructions. Concentration and purity were determined using a GeneQuant™ spectrophotometer (GE Healthcare, Buckinghamshire, UK) and integrity was investigated using a 2100 Bioanalyzer (Agilent Technologies, Stockport, UK). Complementary deoxyribonucleic acid (cDNA) was synthesized using the high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. RT-qPCR was carried out in triplicate and performed using SYBR® Green PCR master mix (Thermo Fisher Scientific). The gene expression of cathepsin K (CTSK) was evaluated in osteoclast cultures, and the expression of bone markers runt-related transcription factor 2 (RUNX2), osterix (OSX), alkaline phosphatase (ALP) and osteocalcin (OC) were evaluated in osteoblast cultures. The results are reported as target genes normalized by the constitutive gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the $2^{-\Delta\Delta Ct}$ method [21].

2.14. Protein levels

Protein analysis was performed by the western blot assay for BMC-derived osteoclasts after three days of treatment. The total cell lysates were obtained using radioimmunoprecipitation assay buffer (RIPA, Sigma-Aldrich) with a cocktail of protease and phosphatase inhibitors. Protein concentration was determined using a bicinchoninic acid protein assay kit (Sigma-Aldrich). Equal amounts of protein (10 μg) were

loaded to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and further transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% nonfat milk for 1 h at room temperature and incubated with specific antibodies against CTSK overnight at 4 °C. After three washes with Tris-buffered saline, 0.1% Tween 20 (TBS-T, Sigma-Aldrich), the membranes were incubated with appropriate horseradish peroxidase (HRP) and conjugated with secondary antibody solution (Luminata Forte, Millipore, Burlington, MA, USA) for two hours at room temperature. The experiments were carried out in triplicate. Bands were quantified using the ImageJ software. Results were normalized by β-Actin as a loading control. Gels were run under the same experimental conditions.

2.15. Statistical analysis

Data are reported as mean ± standard deviation (SD). The Shapiro-Wilk test was used to evaluate normality and the statistical analysis was performed using the Student *t*-test. Data obtained from all evaluations were processed with GraphPad Prism, version 8.0 (GraphPad Software, San Diego, CA, USA). The level of significance was set at 5% in all statistical analyses.

3. Results

3.1. Dabigatran etexilate solution: pharmacological and analytical aspects

Since experiments were conducted over 3–30 days, the stability of the dabigatran etexilate stock solution was an important analytical aspect. HPLC analysis showed that the stock solution of dabigatran etexilate (100 μg/mL) remained stable for 30 days at 2–8 °C, with < 2% of drug reduction being observed (Supplementary Fig. 1A–C). Moreover, the time course assay (one, two and three days) evaluated by HPLC analysis showed a reduction of dabigatran etexilate peak area in cell culture growth medium, suggesting the conversion of dabigatran etexilate to its active moiety, dabigatran (Supplementary Fig. 2A). The capacity of HPLC analysis to detect the dabigatran etexilate peak in cell culture growth medium was determined before the tests (Supplementary Fig. 2B, C). Accordingly, preliminary tests using dabigatran etexilate added to the cell cultures demonstrated effects on the differentiation and activity of osteoblasts and osteoclasts (data not shown).

3.2. Effects of dabigatran etexilate on osteoclasts in an ex vivo model

We first investigated whether BMCs from mice treated with dabigatran etexilate [at a dose calculated in proportion to that used for human anticoagulant therapy (85.7 μg/day)] preserved the ability to generate osteoclasts, as determined by the analysis of cell differentiation and activity (Fig. 1). No difference in the total number of TRAP-stained osteoclasts was observed (Fig. 1A); however, the treatment significantly reduced the total area of osteoclasts compared to the untreated control group ($p = .0001$) (Fig. 1B–D). Consequently, resorptive activity was also reduced in osteoclasts from treated animals ($p = .04$) (Fig. 1E–G). The gene expression of CTSK ($p = .03$) (Fig. 1H) and its protein expression ($p = .01$) (Fig. 1I, J) were significantly reduced in osteoclasts derived from treated animals.

3.3. Effects of dabigatran etexilate on osteoclasts treated in vitro

To better characterize the effects of dabigatran etexilate, osteoclasts were treated with different concentrations of the drug (1–6 μg/mL) and the effects on cell differentiation were assessed. Among the tested concentrations, dabigatran etexilate at 2–6 μg/mL exhibited a similar pattern of cell differentiation impairment (Supplementary Fig. 3A). Thus, 1 μg/mL and 2 μg/mL were selected as the working standardized concentrations since these concentrations did not interphere with cell viability (Fig. 2A).

Dabigatran etexilate treatment (2 μg/mL) resulted in reduced osteoclast differentiation and function, as confirmed by TRAP staining ($p < .0001$) (Fig. 2B–E) and resorptive activity ($p = .01$) (Fig. 2F–H). Consistently, the treatment also reduced the gene ($p < .05$) (Fig. 2I) and protein expression ($p = .01$) (Fig. 2J, K) of the osteoclast marker CTSK.

To confirm whether the effects of dabigatran etexilate were linked to an active principle, dabigatran was used to treat BMC-derived osteoclasts. At the concentrations of 0.1 μg/mL, 0.3 μg/mL and 3 μg/mL, dabigatran did not influence cell viability (0.1 μg/mL: 112.75 ± 13.67; 0.3 μg/mL: 121.25 ± 9.21; 3 μg/mL: 117.50 ± 10.66; control: 100.00 ± 0.00; $p > .05$). The results of TRAP staining demonstrated that dabigatran (3 μg/mL) significantly reduced the number of TRAP+ cells (3 μg/mL: 34.67 ± 6.02; control: 106.74 ± 16.33; $p = .04$). The doses of 0.1 μg/mL and 0.3 μg/mL had no effect on osteoclast numbers (0.1 μg/mL: 110.30 ± 11.29; 0.3 μg/mL: 146.00 ± 7.37; control: 106.74 ± 66.33; $p > .05$).

3.4. Effects of dabigatran etexilate on osteoblasts

Considering the biological mechanisms of interaction between osteoclasts and osteoblasts in bone remodeling/repair and the impairment observed in osteoclasts, our next step was to assess the effects of dabigatran etexilate on osteoblast cell cultures. The tests were performed on cell cultures of calvaria-derived osteoblasts grown on polystyrene, Ti with nanotopography (Ti-Nano) or Ti with machining (Ti-Machined). Ti-Nano has been previously described as a surface with higher osteogenic potential [22].

In a first set of experiments, the MC3T3-E1 pre-osteoblastic cell line was used to define the working dose of dabigatran etexilate. The analysis of matrix mineralization revealed that all the tested concentrations (1–6 μg/mL) inhibited MC3T3-E1 differentiation (Supplementary Fig. 3B). Concentrations of 3 μg/mL showed a pattern of osteoblast impairment that could make tests unfeasible. Doses of 1 μg/mL and 2 μg/mL resulted in a similar pattern. Thus, 2 μg/mL was selected as the working standardized concentration.

Viability was also tested for calvaria-derived osteoblasts. The MTT assay showed that dabigatran etexilate (2 μg/mL) had no effect on cell viability compared to control. Optical density values were determined as percent control absorbance at three (2 μg/mL: 92.29 ± 5.53; control: 100.00 ± 0.00; $p > .05$) and seven days (2 μg/mL: 91.53 ± 7.68; control: 100.00 ± 0.00; $p > .05$) of treatment.

Dabigatran etexilate (2 μg/mL) treatment inhibited osteoblast differentiation and function in all tested surfaces; in cells grown on polystyrene, on Ti-Machined and on Ti-Nano (Fig. 3). This effect was verified by the reduction of ALP activity of cells grown on polystyrene after seven days of treatment ($p < .0001$) (Fig. 3A-C) and the reduction of mineralized matrix formation ($p < .0001$) (Fig. 3D–F) after 14 days of treatment. In calvaria-derived osteoblasts grown on Ti-Machined, the presence of dabigatran etexilate (2 μg/mL) also reduced ALP activity ($p < .0001$) (Fig. 3G–I) and mineralized matrix formation ($p = .001$) (Fig. 3J–L). Similarly, ALP activity ($p < .0001$) (Fig. 3M–O) and the calcium deposits ($p < .0001$) (Fig. 3P–R) were significantly lower in treated cells grown on Ti-Nano.

Corroborating the phenotypic findings, osteoblasts grown on polystyrene and on both Ti-Machined and Ti-Nano surfaces exhibited reduced gene expression of ALP and OC in the presence of dabigatran etexilate (2 μg/mL) ($p < .0001$) (Fig. 4A–C). No statistically significant difference in the expression of early osteoblast markers, RUNX2 and OSX, was observed between the dabigatran etexilate and control groups.

The active principle, dabigatran, was also used to treat osteoblast cell cultures. Accordingly, inhibition of cell differentiation was also observed. A significant reduction in mineralized matrix formation detected by optical density values of calcium was observed at the highest

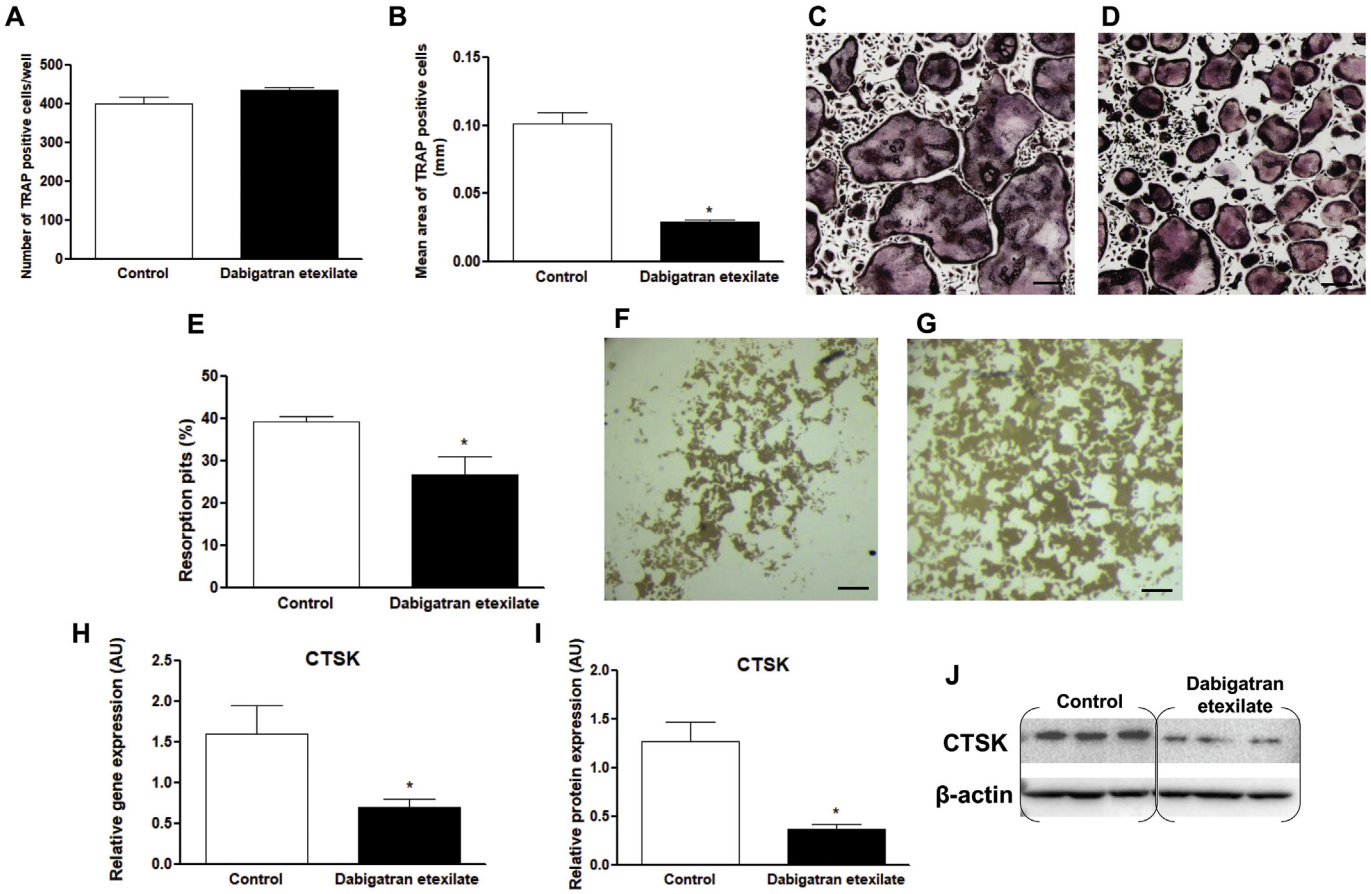

Fig. 1. Assessment of the differentiation of bone marrow cell (BMC)-derived osteoclasts from mice treated with dabigatran etexilate administered by gavage twice daily for 28 days. Number (**A**) and mean area (**B**) of tartrate-resistant acid phosphatase (TRAP)-positive cells identified as osteoclasts after five days of culture. TRAP + cells of non-treated animals (**C**), and of animals treated with dabigatran etexilate (**D**). Resorptive activity was measured by the pit formation area in the osteoassay microplate after 10 days of culture (**E**). Resorption pit of non-treated animals (**F**), and of animals treated with dabigatran etexilate (**G**). For the analysis of gene expression and protein levels of cathepsin K, an osteoclast marker, the total cell lysates were subjected to real-time polymerase chain reaction (**H**) and western blot analysis (**I**, **J**) after three days of treatment. Data are reported as mean and expressed in relation to the control β-Actin and the constitutive gene glycer-aldehyde-3phosphate dehydrogenase (GAPDH), respectively. Statistical analysis was performed by the Student *t*-test. $*p < .05$ compared to control. Acquisition of images with the Cytation™ 5 Cell Imaging Multi-Mode Reader. Bands were quantified using The ImageJ Software and the Leica Application Suite for pit area determination using contouring tools. AU, arbitrary unit; scale bars = 40 μm.

concentrations of dabigatran, *i.e.*, 3 μg/mL and 6 μg/mL, compared to control (3 μg/mL: 0.42 ± 0.09; 6 μg/mL: 0.38 ± 0.07; control: 0.51 ± 0.06; $p < .05$). The doses of 0.1 μg/mL and 0.3 μg/mL had no effect on mineralization (0.1 μg/mL: 0.44 ± 0.08; 0.3 μg/mL: 0.55 ± 0.04; control: 0.51 ± 0.06; $p < .05$).

4. Discussion

Bone healing consists of complex biological mechanisms involving local and systemic factors. The recruitment of progenitor cells and their proliferation and differentiation into osteoblasts and osteoclasts are essential during bone repair [23]. Knowledge about the effects of medications on bone metabolism, in particular postoperatively used drugs, is of clinical relevance. In surgical orthopedic practice, this is particularly applicable since the prescription of drugs for thromboprophylaxis is usually necessary after major procedures. These medications have effects on bone cells, impairing the surgical outcome, which is strictly dependent on bone remodeling [16]. Herein, we evaluated the effects on osteoclast and osteoblast cells of a DTI in two presentations: the mesylate salt form dabigatran etexilate (Pradaxa®) and its active principle dabigatran. Our main findings showed that both dabigatran and Pradaxa® inhibited osteoclast differentiation and resorptive activity in a dose and time-dependent manner, were also negatively affected, as demonstrated by the reduced expression of ALP and OC, as well as the shortened ALP activity and mineralized matrix formation. Particularly, osteoblasts growing under favorable osteogenic conditions such as on a Ti-Nano surface, still showed impairment of their differentiation ability and function.

Osteopenia, a recognized side effect of long-term therapy with heparin and low-molecular-weight heparin, may impair the healing of bone fractures and the osseointegration of prostheses [1,10,11]. Therefore, several studies have investigated the effects of anticoagulants on cell differentiation and function, as well as on bone structure [7–9,11,13–16]; however, the specific effects of these drugs on the complex mechanism of bone healing remain unclear. Previous *in vitro* experiments have tested the impact of several anticoagulants on bone cells. These experiments have focused on heparin-related drugs [7,8,24–26] and, more recently, on DOACs [11,13,15,17]. Nevertheless, no data on the effects of dabigatran on cell cultures have been described in the literature thus far.

Regarding the impact of anticoagulant therapy on cultured osteoclasts, while data about DOACs are not available, the heparin-related impairment has been well described. In a co-culture system, heparin enhanced osteoclastic activity but did not change osteoclastogenesis [7]. Muir et al. [26] have observed that heparin increased bone resorption by inflating both osteoclast number and activity. Conversely, Folwarczna et al. [24] have shown that heparins affected osteoclast formation in rat BMCs in two directions depending on the drug

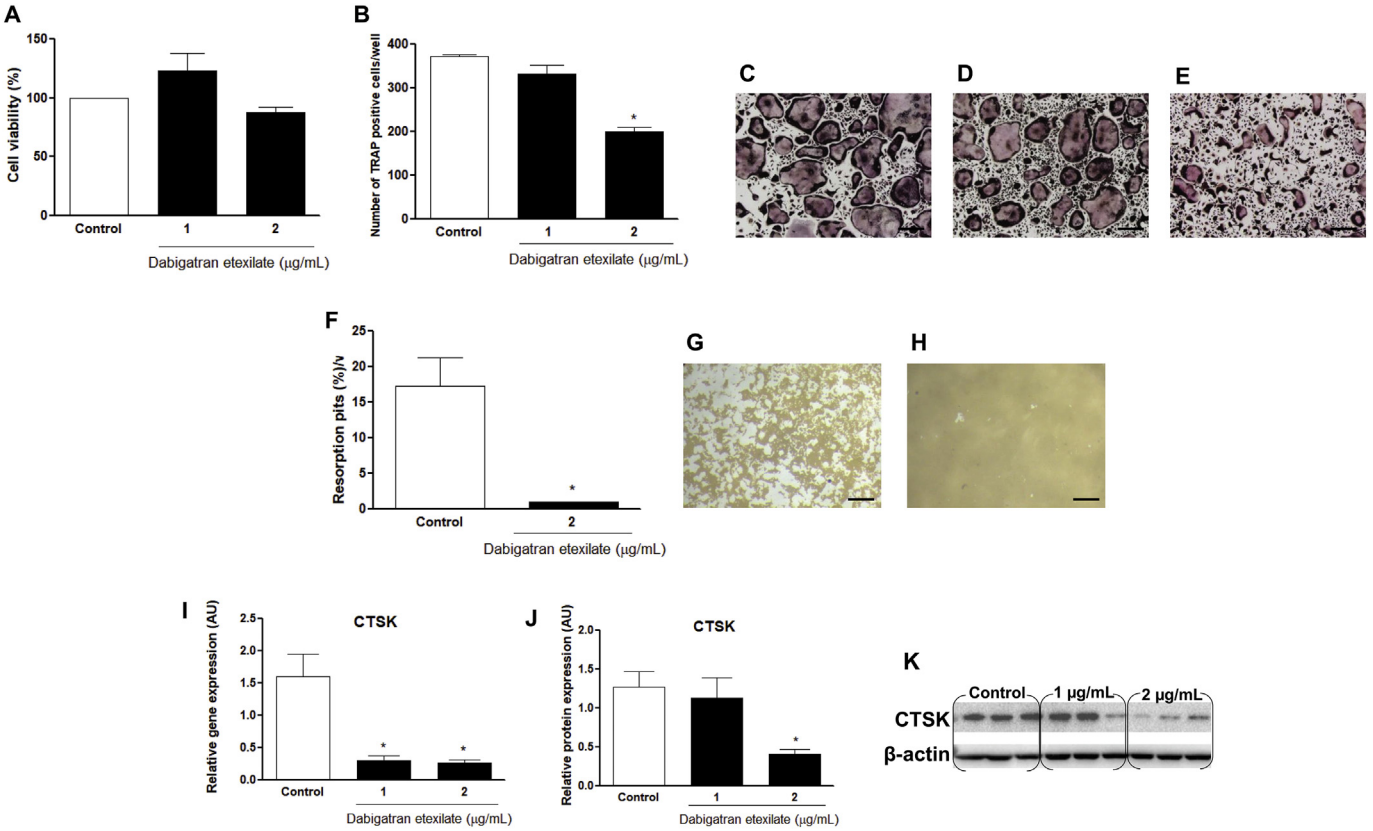

Fig. 2. Effect of dabigatran etexilate (1 μg/mL and 2 μg/mL) on the viability and differentiation of bone marrow cell (BMC)-derived osteoclasts. Results of the optical density values of the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay at three days of treatment expressed as percentage relative to the absorbance of the control (**A**). Number of tartrate-resistant acid phosphatase (TRAP)-positive cells after five days of culture (**B**). TRAP + cells of the control group (**C**) and of the group treated with dabigatran etexilate at 1 μg/mL (**D**) and 2 μg/mL (**E**). Resorptive activity was measured by the pit formation area after 10 days of treatment (**F**). Resorption pit of the control group (**G**) and of the group treated with dabigatran etexilate at 2 μg/mL (**H**). Gene (**I**) and protein expression (**J**, **K**) of cathepsin K (CTSK) after three days of treatment. Results were reported in relation to the control β-Actin and to the constitutive gene glycer-aldehyde-3-phosphate dehydrogenase (GAPDH), respectively. Data are reported as mean. Statistical analysis was performed by the Student *t*-test. *$p < .05$ compared to control. AU, arbitrary unit; scale bars = 40 μm.

concentration. At the highest concentrations, heparins reduced the number of osteoclasts and at lower concentrations they increased osteoclast formation [24]. In the present study, dabigatran and the prodrug dabigatran etexilate reduced osteoclast differentiation at their highest concentrations, *i.e.*, 2 μg/mL and 3 μg/mL. The resorption pit assay also showed reduction of function in the treated group. Furthermore, dabigatran etexilate downregulated the expression of CTSK, a key marker of osteoclast differentiation and activity. Consistent with our *in vitro* data, we also observed that the differentiation and function of osteoclasts was impaired in mice treated with dabigatran etexilate. Nevertheless, when dabigatran-treated rats were compared to warfarin-treated rats or non-treated animals it was observed that dabigatran have no major deleterious effects on bone structural parameters [12,27].

In vitro effects of DOACs have been mostly reported in experiments with osteoblasts [11,13,15,17]. Somjen et al. [15] have shown that rivaroxaban at different concentrations (0.01–50 μg/mL) inhibited the proliferation of osteoblasts from female individuals. This effect was maintained even in the presence of molecules that stimulate DNA synthesis and ALP specific activity [15]. Similarly, in the present investigation, the upregulation of osteoblast differentiation markers induced by Ti-Nano [28–31] was inhibited in the presence of dabigatran etexilate.

The effect of melagatran, a DTI similar to dabigatran, was investigated in human osteoblasts [13]. In line with our findings, melagatran at the highest concentration (50 nmol/mL) caused a significant reduction of collagen type I deposition and ALP activity [13]. The inhibition of ALP activity by DOACs was also confirmed in two previous studies [11,17] in which rivaroxaban induced a reduction of ALP activity in osteoblast cultures. However, in one study [11], the osteoblast function remained unaffected. In the other study [17], rivaroxaban and enoxaparin treatment led to a reduction in bone morphogenetic protein-2 (BMP-2), OC and RUNX2 mRNA expression. In contrast, in our experiment, there was no change in RUNX2 or OSX expression after dabigatran etexilate treatment.

Although the exact mechanism of interaction between dabigatran etexilate and bone cells remains unclear, the physiological role of thrombin on bone metabolism was previously investigated [18,32]. Considering the thrombin inhibitory effect of dabigatran, these finding suggest how the drug affects the differentiation and activity of osteoclasts and osteoblasts. The thrombin/PAR-1 system was related to mediate many, but not all, of the thrombin effects on bone cells [18]. The system maintains normal bone remodeling by activating the receptor activator of RANKL and limiting osteoprotegerin (OPG) synthesis by osteoblasts [32]. Moreover, the catalytically active thrombin stimulates the expression of the plasminogen activator inhibitor-1 (PAI-1) and prostaglandins (PGs), especially PGE2 and interleukin-6 (IL-6) by osteoblasts [18] increasing RANKL/OPG ratio.

Sivagurunathan et al. [33] demonstrated that thrombin does cause an increase in the ratio of RANKL/OPG thereby stimulate osteoclast differentiation mixed cultures of osteoblastic cells and osteoclast precursors. However, experiments with mouse bone marrow stromal cells demonstrated that thrombin had the opposite effect inhibiting osteoclast differentiation. Thus, thrombin probably acts in the early stages of

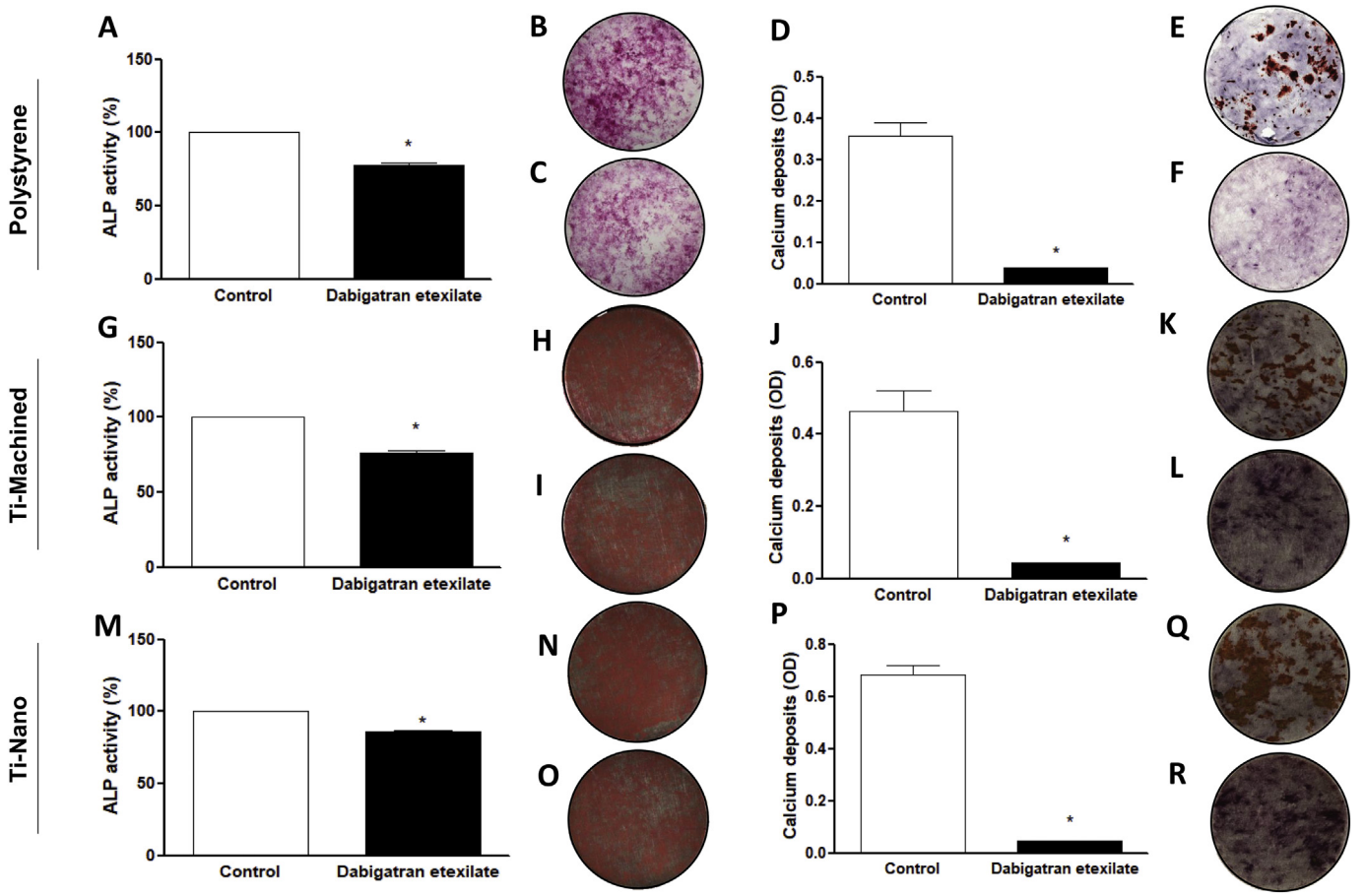

Fig. 3. Effect of dabigatran etexilate (2 μg/mL) on the differentiation of calvaria-derived osteoblasts grown on polystyrene, titanium (Ti) with nanotopography (Ti-Nano) or machined Ti (Ti-Machined). *In situ* alkaline phosphatase (ALP) activity measured by percent stained area after seven days of treatment of cells grown on polystyrene (**A**). Stained area of *in situ* ALP activity of the control group (**B**) and of the dabigatran etexilate group (**C**). Extracellular matrix mineralization measured by optical density (OD) values of calcium deposit mineralization after 14 days of treatment of cells grown on polystyrene (**D**). Alizarin red-stained calcium deposits in the control group (**E**) and in the dabigatran etexilate group (**F**). *In situ* ALP activity of cells grown on Ti-Machined (**G**), stained ALP area in the control group (**H**) and in the dabigatran etexilate group (**I**). Extracellular matrix mineralization of cells grown on Ti-Machined (**J**), alizarin red-stained calcium deposits in the control group (**K**) and the dabigatran etexilate group (**L**). *In situ* ALP activity of cells grown on Ti-Nano (**M**), stained ALP area in the control group (**N**) and in the dabigatran etexilate group (**O**). Extracellular matrix mineralization of cells grown on Ti-Nano (**P**), alizarin red-stained calcium deposits in the control group (**Q**) and in the dabigatran etexilate group (**R**). Statistical analysis was performed by the Student *t*-test. $*p < .05$ compared to control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

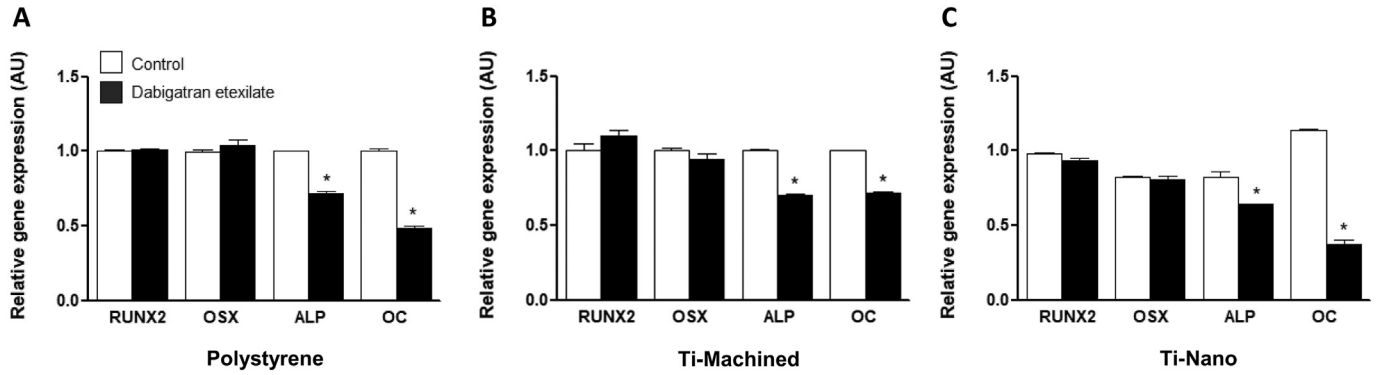

Fig. 4. Gene expression of the bone markers runt-related transcription factor 2 (RUNX2), osterix (OSX), alkaline phosphatase (ALP), and osteocalcin (OC) in calvaria-derived osteoblasts treated with dabigatran etexilate (2 μg/mL) and grown on polystyrene (**A**), machined titanium (Ti-Machined) (**B**) or Ti with nanotopography (Ti-Nano) (**C**). Data are reported as mean and expressed as fold change in relation to the constitutive gene glycer-aldehyde-3-phosphate dehydrogenase (GAPDH). Statistical analysis was performed by the Student *t*-test. $*p < .05$ indicating a statistically significant difference between control and dabigatran etexilate (2 μg/mL) for each gene evaluated gene. AU, arbitrary unit.

RANKL-induced osteoclast differentiation, directly on osteoclast precursors, and the PAR-1 receptor does not mediate this thrombin's effect on osteoclast precursors [33]. Our findings suggest that the thrombin inhibitor, dabigatran etexilate, might interact with the osteoclasts and osteoblasts precursors resulting in a similar inhibitory effect as observed in primary cells cultured.

Experimental *in vivo* models have been proposed in order to explain how these agents may impair healing of fractures or may cause bone loss, [12,14,16,26,27]. In a histomorphometric analysis comparing warfarin-treated rats and dabigatran-treated animals, an increase in osteoclast activity was observed when warfarin was used [12]. In a study comparing the impact of dabigatran etexilate on male and female mice, no significant negative effects of the drug were observed on bone density, bone strength and bone microstructure when compared to controls [27]. Klüter et al. [16] have demonstrated that there was no significant differences between the control and rivaroxaban groups in femur bone healing. Despite inconclusive results from the limited number of *in vivo* models with DOACs, there is a consensus from *in vivo* studies on bone metabolism that heparins reduce bone mineral mass [34] and increase bone resorption, as a consequence of the increase of both osteoclast number and activity [26]. The effects on osteoblasts, such as the inhibition of ALP activity and the impairment of OC carboxylation have been demonstrated in rats treated with heparin [26] and warfarin [14], thus strengthening the *in vitro* findings [17].

5. Conclusions

In summary, the findings of our *in vitro* and *ex vivo* models clearly demonstrated for the first time the negative impact of the prodrug dabigatran etexilate and its active principle, dabigatran, on osteoblasts and osteoclasts. Further *in vivo* drug-comparative studies are encouraged to disclose the response of the set of cells in tissues or organs. The present study provides new evidence about the potential negative impact of dabigatran etexilate on bone turnover and repair.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.thromres.2019.12.014.

Author contributions

A.L. Rocha, R.L.B. Trevisan, L.F. Duffles, J.A.A. de Arruda, S. Macari, I.M.A. Diniz, M.M. Beloti, A.L. Rosa, S.Y. Fukada, G.A.C. Goulart, D.D. Ribeiro, and T.A. Silva contributed to the conception and design of the study, to data acquisition, analysis, and interpretation, and drafted and critically revised the manuscript. T.A. Silva, A.L. Rocha, T.M. Taira, B.R.D. Assis, J.A.A. de Arruda, and L.G. Abreu contributed to the conception and design of the study and to data acquisition, and drafted and critically revised the manuscript. All authors gave final approval and have agreed to be accountable for all aspects of the work.

Declaration of competing interest

None.

Acknowledgments

We wish to thank Mr. Roger R. Fernandes and Ms. Fabiola S. de Oliveira for technical assistance during the experiments. The Centro de Aquisição e Processamento de Imagens - CAPI (https://www2.icb.ufmg.br/capi/) at the Universidade Federal de Minas Gerais, Brazil is also acknowledged for providing the equipment and technical support for experiments involving data collection and image acquisition. The authors thank the Coordination for the Improvement of Higher Education Personnel (CAPES, Finance Code 001), Brazil. A.L.R. and J.A.A.A. are the recipients of scholarships. Mrs. E. Greene provided English editing of the manuscript. The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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