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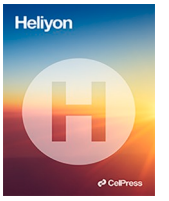

Research article

Bevacizumab, a vascular endothelial growth factor inhibitor, promotes orthodontic tooth movement in an experimental rat model

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ABSTRACT

Objective: This study aimed to evaluate the impact of bevacizumab on orthodontic tooth movement (OTM) in Wistar rats.
Materials and methods: The OTM model was constructed by placing an orthodontic coil spring between the maxillary first molar and anterior tooth. Bevacizumab (Avastin®; 10 mg/kg twice per week) was started one week before the OTM and continued for 3 weeks. After 1 and 2 weeks, OTM distance and anterior tooth mobility were measured. Thereafter, the maxilla was dissected for micro-CT microarchitectural analysis, followed by histological analysis, and tartrate-resistant acid phosphatase (TRAP) staining. Moreover, the distributions of collagen fibers type-I and –III (Col-I and Col-III) were evaluated using Picro-Sirius red staining.
Results: Orthodontic force prompted bone resorption and formation on the pressure and tension sides, respectively. Bevacizumab therapy resulted in a 42% increase of OTM, particularly after 2 weeks. Furthermore, bevacizumab disturbed the morphometric structure at both pressure and tension sites. The histological evaluation indicated about 35–44% fewer osteoblasts in the bevacizumab group, especially at the tension side, whereas the proportion of TRAP-positive osteoclasts at the pressure side was 34–37% higher than the control. The mature Col-I was reduced at the tension site by 33%, whereas the Col-III/Col-I ratio was enhanced by 20–44% at pressure and tension sites, after 2 weeks, in the bevacizumab group.
Conclusion: Anti-vascular bevacizumab therapy accentuates OTM in rat model, possibly through the enhancement of bone resorption, at the pressure side, and the reduction of bone formation, at the tension side as well as dysregulation of collagen fibers distribution.

1. Introduction

Orthodontic mechanical force is a key procedure to achieve the finest occlusal relationship through formation of simultaneous

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pressure and tension sites between the tooth and the alveolar bone leading to orthodontic tooth movement (OTM). The pressure site is characterized by an enhanced osteoclastic activity, while at the opposite tension site, osteoblastic bone formation activity is promoted [1]. Therefore, factors, such as medications, which influence osteoblastogenesis and/or osteoclastogenesis may have an impact on OTM.

The process of bone regeneration and remodeling relays on skeletal vasculature and blood flow [2,3]. It is well-known that skeletal blood flow plays a vital role in bone homeostasis and repair [3]. Poor vascular function may lead to impaired skeletal blood flow, which may negatively affect the endothelial cells in the inner bone moiety, leading to defective angiogenesis and osteogenesis. Vascular endothelial growth factor (VEGF) is a key mediator of angiogenesis that binds to VEGF receptors expressed on vascular endothelial cells, promoting their migration, differentiation and survival as well as enhancing vascular permeability. Moreover, it has been shown that VEGF overexpression in osteoblasts enhances the number of blood vessels and increases the bone mass in skeletal sites [4]. VEGF was also found to trigger osteoblast development and alkaline phosphatase release [5].

Recently, targeted chemotherapies has gained attention as they specifically target the tumor cells to block oncogenic pathways [6] and/or selected growth factors to restrict new vasculature formation [7]. The antiangiogenic medication bevacizumab (Avastin®) is an example of targeted mono-chemotherapy for several malignancies. Bevacizumab is a monoclonal antibody, which selectively targets VEGF-A to prevent binding with its specific receptors. Studies suggested that *anti*-VEGF therapies might negatively impact bone healing and remodeling. For instance, the experimental antiangiogenic medication, TNP-470, decreased the bone formation rate in the peri-implant tissues [8]. Another medication, ranibizumab, compromised the skeletal healing and osseointegration of dental implants in rat [9]. Furthermore, our previous work showed that bevacizumab delays the extraction socket healing [10] and reduces implant osseointegration in rabbit models [10,11].

As the number of adult patients going for orthodontic treatments is increasing and the clinical application of bevacizumab is rising, it is important to explore the role of such *anti*-VEGF medication on OTM. Given the fact that the VEGF receptors are expressed in both osteoblasts and osteoclasts, suggesting its role in regulating their activities [12] and also the importance of the skeletal vasculature and angiogenesis for bone remodeling, it can be anticipated that the anti-vascular therapy may have an impact on alveolar bone remodeling in response to orthodontic mechanical force. Consequently, it can be hypothesized that the *anti*-VEGF medication, bevacizumab, will interfere with the angiogenic process during orthodontic force application, thereby affecting the bone formation and remodeling at the pressure and tension sites. Therefore, the present study aimed to investigate the impact of bevacizumab on bone remodeling, at the pressure and tension sites, during OTM using a well-established experimental rat model.

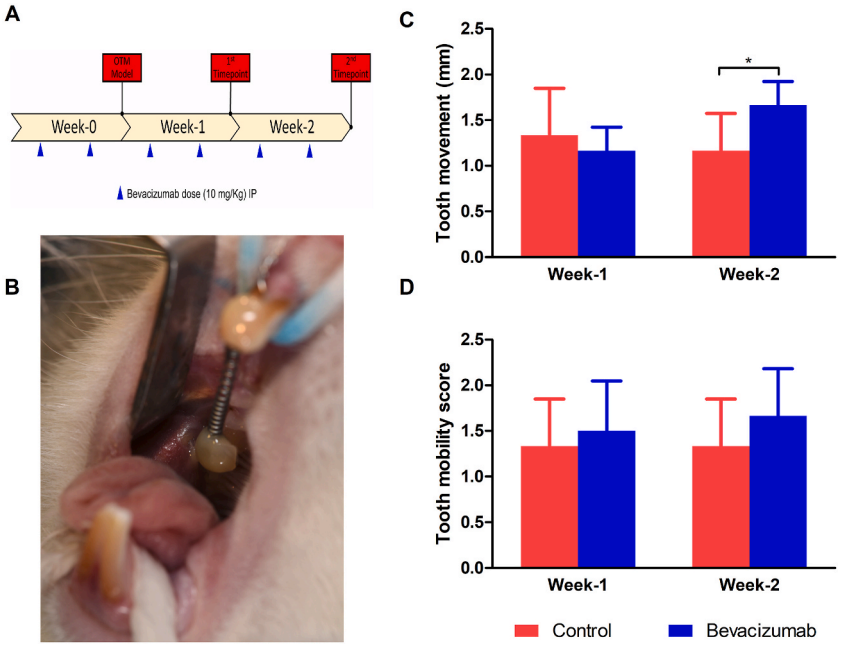

Figure-1. Experimental model and clinical assessments. (A) The timeline of the experiment indicating the course of bevacizumab dosing (10 mg/kg IP twice per week) starting one week before the animal model. (B) The orthodontic tooth movement (OTM) model in Wistar albino rats using a 4.0-mm NiTi coil spring (30–35 g force) between the right central incisor and the first maxillary molar. (C) Tooth movement as indicated by the distance difference (mm) between 1st molar and incisor before OTM model (baseline) and after 1 and 2 weeks of the orthodontic force application. (D) Right incisor mobility score after 1 and 2 weeks of orthodontic force application. Numerical data is presented as Mean ± SD (n = 6) and significance is presented as * $p < 0.05$.

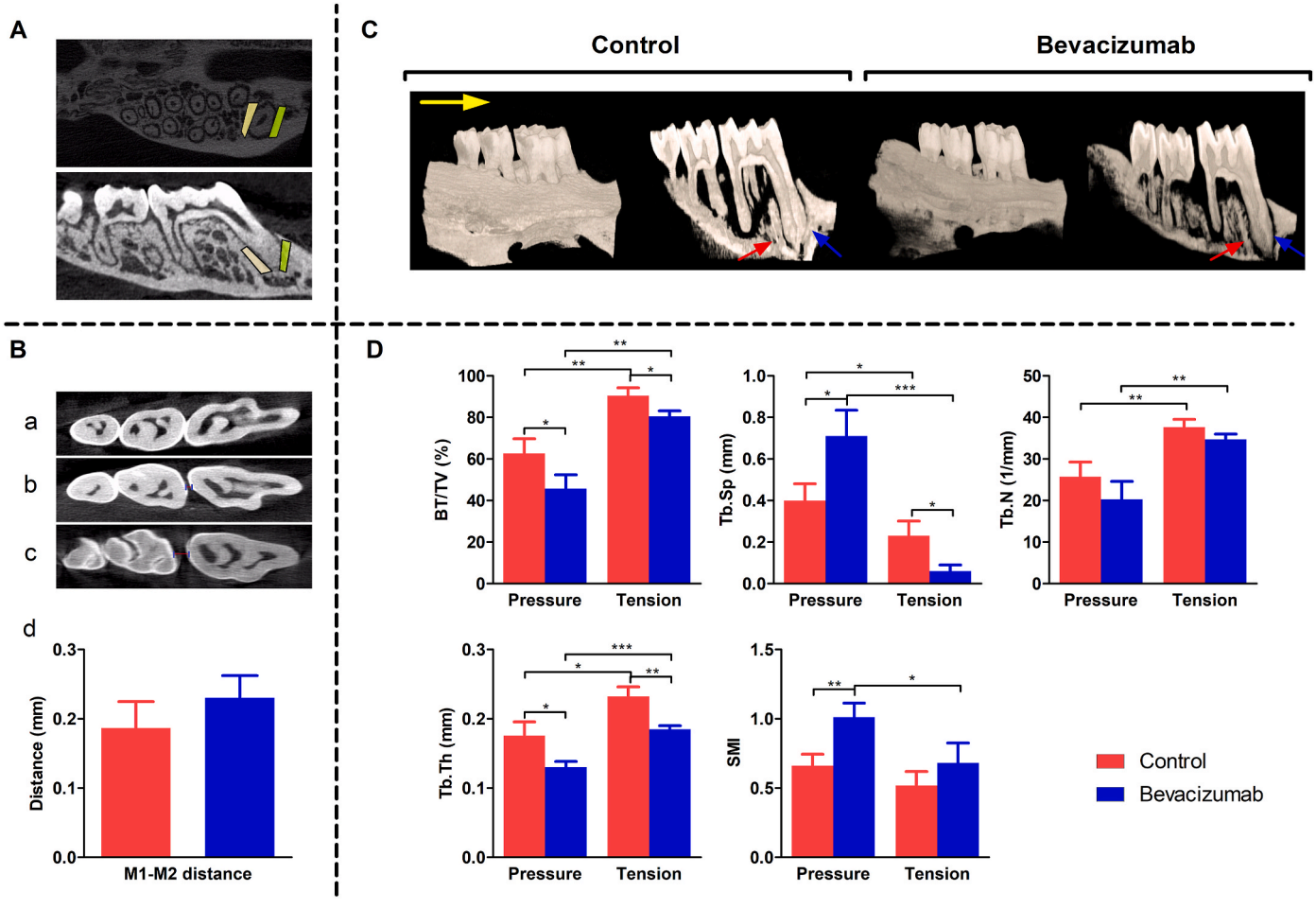

Figure-2. Measurement bone of microarchitecture by Micro-CT. (A) Micro-CT images showing the top and coronal views of the two ROIs at pressure (green) and tension (yellow) sites. (B) Top view of the three maxillary molars showing (a) no tooth movement, (b) tooth movement in control group, (c) tooth movement in bevacizumab group, and (d) distance (mm) between M1 and M2. (C) 3D micro-CT representative images from the two experimental groups showing the coronal view of the right maxilla molars after application of orthodontic force for 2 weeks. Red and blue arrows point to the ROIs at the tension and pressure sites, respectively, while the yellow arrow denotes orthodontic force direction. (D) Bone morphometric analysis after micro-CT scanning at the pressure and tension sites: Bone volume fraction (BV/TV; %), Trabecular separation (Tb.Sp; mm), Trabecular number (Tb.N; 1/mm), Trabecular Thickness (Tb.Th; mm), and Structure model index (SMI). Numerical data is presented as Mean ± SD (n = 6) and significance is presented as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2. Materials and methods

Ethical approval and sample calculations: In this study, twenty-four male Wistar albino rats (250–300 g) were utilized. The sample size was estimated using G*Power for the repeated measure within-between interaction using the following criteria: effect size (f) = 0.25, alpha error probability (α) = 0.05, and power (1-b) = 0.9. Animals were kindly provided by the Animal House facility. Experimental procedures followed the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals (8th edition-2011) and adapted ARRIVE guidelines. Animals were provided free access to water and food, *ad libitum*, in a pathogen- and stress-free environment at 24 °C, under a light-dark cycle. The study was approved by the institutional review board (IRB# 2020-02-301).

Animal model and study design: Animals were randomly assigned into two groups: (A) control group and (B) test, bevacizumab, group (n = 12 per group). The control group received saline injection intraperitoneally at the same time as bevacizumab administration in the test group. Bevacizumab (Avastin®; Roche Pharma AG, Basel, Switzerland) was administered intraperitoneally in a dose of 10 mg/kg twice per week, starting one week before OTM procedure **(Figure-1 A)** [13]. For OTM procedure, rats were anesthetized with xylazine (5 mg/kg) and ketamine (60 mg/kg) intraperitoneally. Afterwards, a nickel-titanium orthodontic appliance with a closed-coil spring, releasing 30–35 g of force, was bonded by light-cured resin between the maxillary right 1st molar and the maxillary right incisor, by two specialist orthodontists, who were blinded to the experimental groups **(Figure-1 B)**. The orthodontic appliance was inspected daily, and any detached or loose-fitting coil was replaced. Six animals from each group were sacrificed after one and two weeks of the OTM procedure using an overdose of sevoflurane general anesthesia (Sevorane, Aesica Queenborough Ltd. Queenborough, Kent, UK). After macroscopic evaluation of tooth movement and mobility in each animal, the right maxilla was dissected, cleaned, and fixed in 10% neutral buffered formalin.

Evaluation of tooth movement and mobility: Clinical tooth movement was measured using a mm-scaled periodontal probe (UNC-15 probe). The gap (in mm) between the most mesial surface of the maxillary right first molar and the maxillary right incisor was recorded at baseline (at the start of OTM) and after each evaluation time point (1 & 2 weeks). The difference between the initial (baseline) and final (at the time of sacrificing) readings was calculated. In addition, mobility grades were recorded for the anterior tooth at the time of sacrificing according to Vasconcelos et al. study [14] with minor modifications as follows: (0) physiological mobility, (1) slight vestibule-oral mobility, (2) relative mesial-distal and vestibular-palatal mobility, and (3) critical vertical mobility.

Morphometric analysis using Micro-CT: A high-resolution micro-CT system (SkyScan 1172, Bruker micro-CT, Kontich, Belgium) was used. After 2 weeks of orthodontic force application, the dissected maxilla samples were scanned, on the right side, by applying the following parameters: slice thickness of 0.014 mm, voltage of 100 kV, and electrical current of 100 μA. Next, a reconstruction and analysis processes were conducted by NRecon and CTAn software (Bruker micro-CT, Belgium). The horizontal offset of the 1st molar was considered as the distance between the most mesial point of the 2nd molar and the most distal point of the 1st first molar. Furthermore, two regions of interest (ROIs) were considered **(Figure-2 A)** around the mesial root of the first molar as follows; (A) the pressure site (the mesial coronal alveolar bone to the root) and (B) tension site (the distal coronal alveolar bone to the root). Major morphometric parameters of the alveolar bone microarchitecture were calculated at each ROI, defined as follows: bone volume/tissue volume (BV/TV) percentage; trabecular thickness (Tb.Th) (mm); Trabecular number (Tb.N) (mm− 1); trabecular spacing (Tb.Sp) (mm); structure model index (SMI).

Histological investigation: After micro-CT scanning, each right maxillary segment was fixed in 10% neutral buffered formalin. Samples were firstly decalcified in Shandon TBD-1 decalcifier (Cat# 6764001, Thermo Scientific, USA) for 4 days, and subsequently embedded in paraffin and sectioned into 5 μm sections using a microtome (Accu-Cut® SRM™ 200 rotary microtome, Sakura Finetek Inc., Torrance, CA, USA). Slides were stained with hematoxylin and eosin (H&E) stain (Cat # H-3502, Vector Laboratories Inc., Mowry Ave Newark, CA, United States) and evaluated blindly under 200× magnifications using an inverted microscope (Nikon Eclipse Ts2R, Nikon Instruments Inc., Melville, NY, USA). Slides were evaluated semi-qualitatively for the number of osteoclasts, osteoblasts, and capillaries by a single investigator in a blind manner as previously described [15,16]. Osteoclasts were identified as multiple nucleated cells residing on the surface of bone or resorptive lacunae, while osteoblasts were identified as wide cuboidal cells present at active bone formation area. The blood capillaries count was estimated by calculating the veining (3–5 μm in diameter).

Tartrate resistant acid phosphatase (TRAP) stain: Paraffin blocks were cut into 5 μm sections. After overnight deparaffinization by xylene and rehydration descending ethanol concentrations (100%, 95%, 70%, and 50%), wet sections were fixed in a fixative mixture (for 30 s) consisting of citrate solution (25 ml), acetone (65 ml) and 37% formaldehyde (8 ml). Afterwards, slides were rinsed thoroughly in deionized water and stained using TRAP stain at 37 °C for 1 h as instructed by the kit (Cat # TRAP kit 387, Sigma-Aldrich, St. Louis, MO, USA). TRAP stain solution was prepared by gentle mixing of tartrate solution (provided by the kit) with prewarmed deionized water (37 °C), diazotized fast garnet GBC solution, Naphthol AS-Bl phosphate solution, and acetate solution for 2 min. Slides were then counterstained in hematoxylin solution for 2 min. Slides were inspected blindly under an inverted microscope (Nikon Eclipse Ts2R, Nikon Instruments Inc., Melville, NY, USA) at 200× magnification. Next, slides were semi-qualitatively assessed for the number of $TRAP^{+}$ cells.

Collagen fibers remodeling and distribution: Paraffin blocks were cut into 5 μm sections, deparaffinized and rehydrated as described above. Thereafter, the sections were stained in Picro-Sirius red (Cat # HB6179, Hello Bio, Bristol, UK) for 60 min at room temperature. Subsequently, sections were washed with acidulated water with acetic acid for 1 min. Slides were inspected after mounting under polarized light microscope at 40× magnification (Nikon Eclipse Ts2R, Nikon Instruments Inc., Melville, NY, USA). The mature collagen type I (Col-I) was visualized in red (yellow-orange birefringence), while the immature collagen type III (Col-III) appeared as green birefringence. Semi-quantification of mature and immature (Col-I and Col-III) fibers were preformed using a color histogram function in the Image J 1.37b image analysis system (National Institutes of Health, Bethesda, MD, USA). At each pressure or

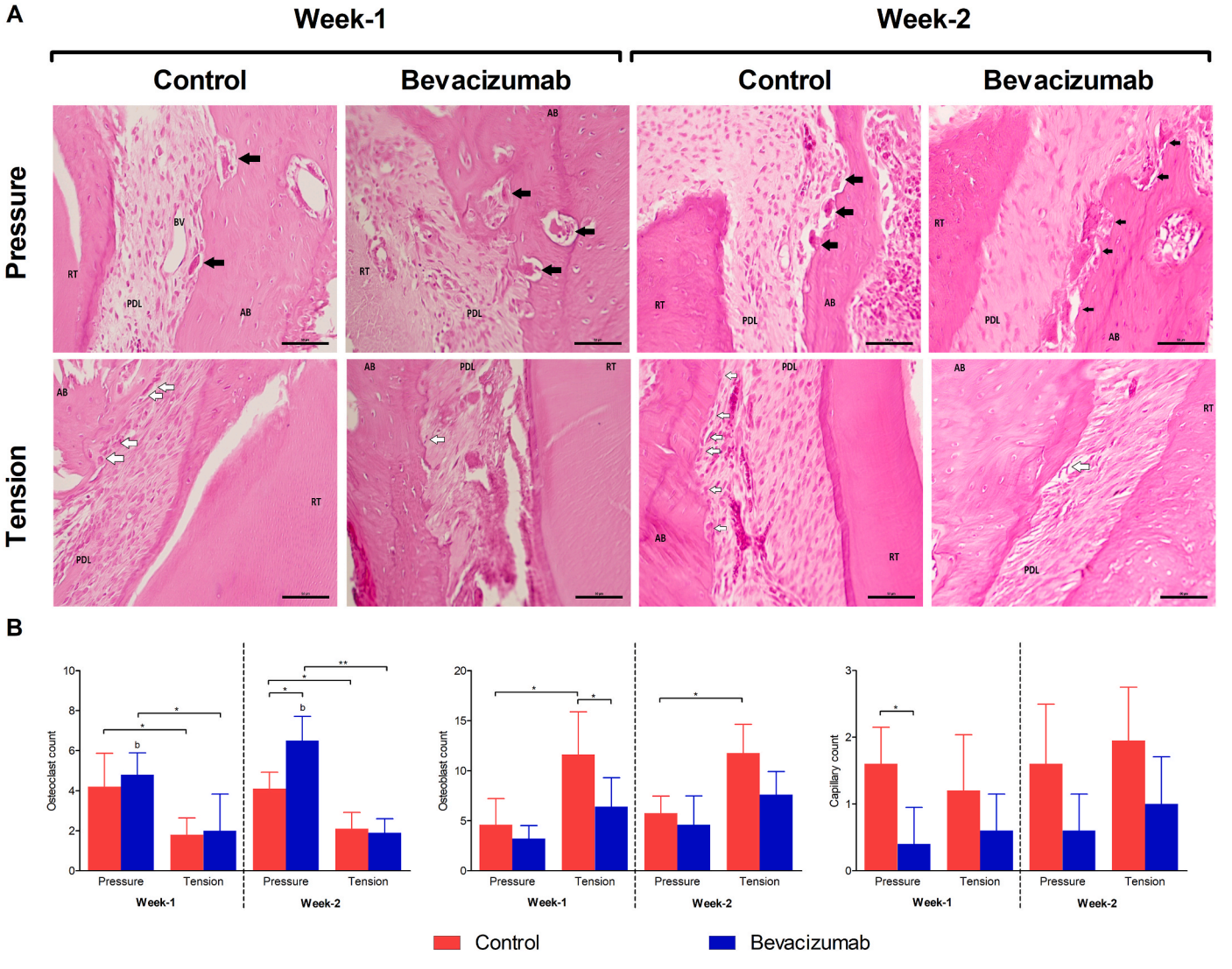

Figure-3. Histopathological evaluation at pressure and tension sites. (A) Representative hematoxylin and eosin sections of the first molar of the right maxilla from control and bevacizumab groups at the tension and pressure sides after 1 and 2 weeks of orthodontic force application. Slides were inspected under magnification of 200× (scale bar = 50 μm). Micrographs revealed the osteoclasts (black arrows), osteoblasts (white arrows), blood capillaries (BV), alveolar bone (AB), periodontal ligaments (PDL), and root (RT) at the tension and pressure sides (B) Semi-quantification of osteoclasts, osteoblasts, and capillaries numbers at the tension and pressure sides after 1 and 2 weeks of the orthodontic force application. Numerical data is presented as Mean ± SD (n = 6) and significance is presented as * $p < 0.05$ and ** $p < 0.01$. Similar small letters indicate significance ($p < 0.05$) between timepoints within the same group and site.

tension site, 3 ROIs (200 × 200 μm/each) were chosen at the apical, central, and cervical areas. The mean value of the 3 ROIs at each site was considered.

Statistical analysis: Numerical data is presented as Mean ± SD. Statistical tests were performed using Graph Pad Prism (version 5) software (GraphPad Software, Inc., La Jolla, CA, USA). Two-way ANOVA followed by Bonferroni post-hoc test was used to compare different groups at the pressure and tension sites. The Mann–Whitney *U* test compared the change at different timepoints within the same site and group. Significance was considered at $p < 0.05$. For the semi-quantitative evaluation, the intraclass correlation coefficient (ICC) was estimated to confirm intra-rater reliability.

3. Results

After 2 weeks of OTM, bevacizumab significantly ($p < 0.05$) increased OTM compared to control group **(Figure-1 C)**. In addition, tooth mobility was relatively higher in the bevacizumab group after 1 and 2 weeks of OTM, but the differences were not statistically significant **(Figure-1 D)**. Micro-CT imaging revealed that the distance between the 1st and 2nd molars was wider in bevacizumab group than control group after 2 weeks of OTM **(Figure-2 B [a-d] & C)**. For the microstructural parameters, bevacizumab significantly reduced BV/TV percentage ($p < 0.05$) and Tb.Th ($p < 0.05$) both in the pressure and tension sites. In contrast, the Tb.Sp parameter although was significantly increased by the bevacizumab ($p < 0.05$) at the pressure site, it was significantly reduced at tension site. The SMI parameter was significantly ($p < 0.01$) higher in bevacizumab *vs*. control group at the pressure site. The pressure sites demonstrated significantly lower BV/TV% ($p < 0.01$), Tb.N ($p < 0.01$), and Tb.Th ($p < 0.05$) and a higher Tb.Sp ($p < 0.05$) in contrast to the tension sites irrespective of bevacizumab treatment **(Figure-2 D)**.

The histological evaluation revealed evident bone formation and bone resorption activities at the tension and pressure sites, respectively **(Figure-3 A)**. At the pressure site, a significantly ($p < 0.05$) higher osteoclasts count was reported in control and bevacizumab groups compared to tension side. Notably, bevacizumab significantly ($p < 0.05$) increased osteoclasts count at the pressure side compared to control animals after 2 weeks. On the other hand, bevacizumab significantly ($p < 0.05$) lowered the osteoblast count at the tension side compared to the control group, particularly at the early timepoint. Moreover, bevacizumab limited the blood capillaries count at the pressure and tension sides compared to the control animals, particularly at the pressure side of the early

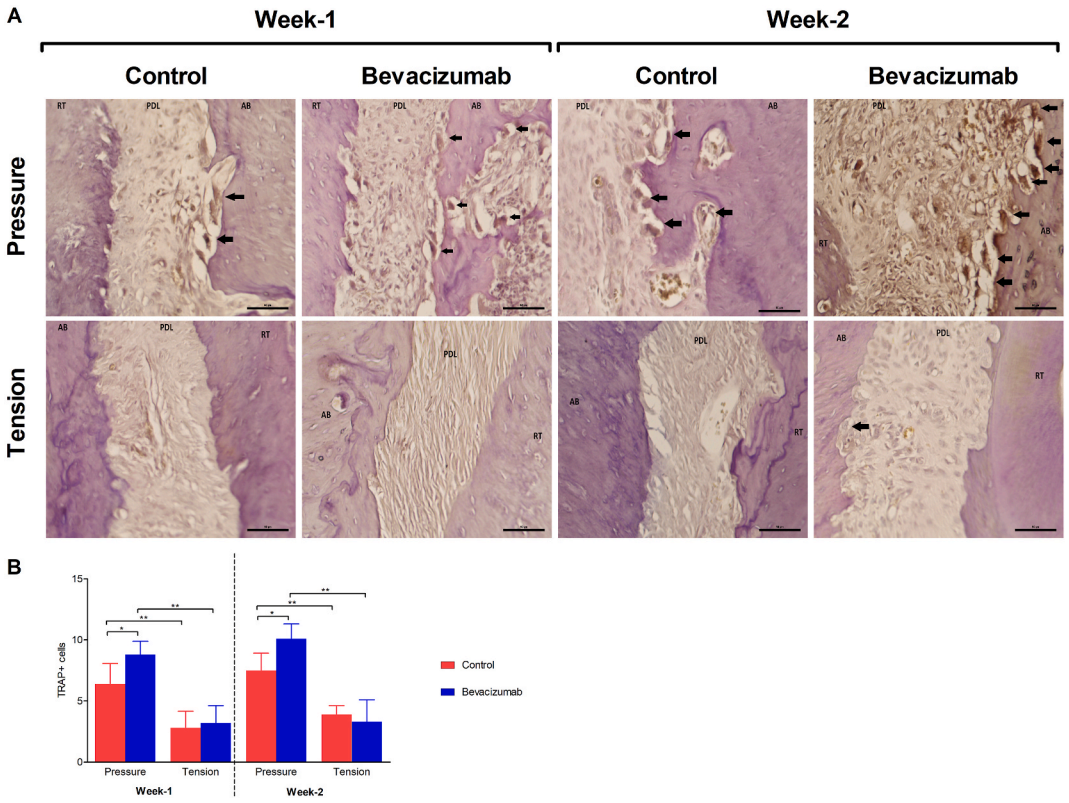

Figure-4. Evaluation of TRAP$^+$ cells at pressure and tension sites. (A) Representative sections of the first molar of the right maxilla from control and bevacizumab groups after 1 and 2 weeks of the orthodontic force application. Slides were inspected under magnification of 200× (scale bar = 50 μm). Micrographs revealed the TRAP$^+$ cells (black arrows), alveolar bone (AB), periodontal ligaments (PDL), and root (RT) at the tension and pressure sides. (B) Semi-quantification of the TRAP$^+$ cells at the tension and pressure sides after 1 and 2 weeks of the orthodontic force application. Numerical data is presented as Mean ± SD (n = 6) and significance is presented as * $p < 0.05$ and ** $p < 0.01$.

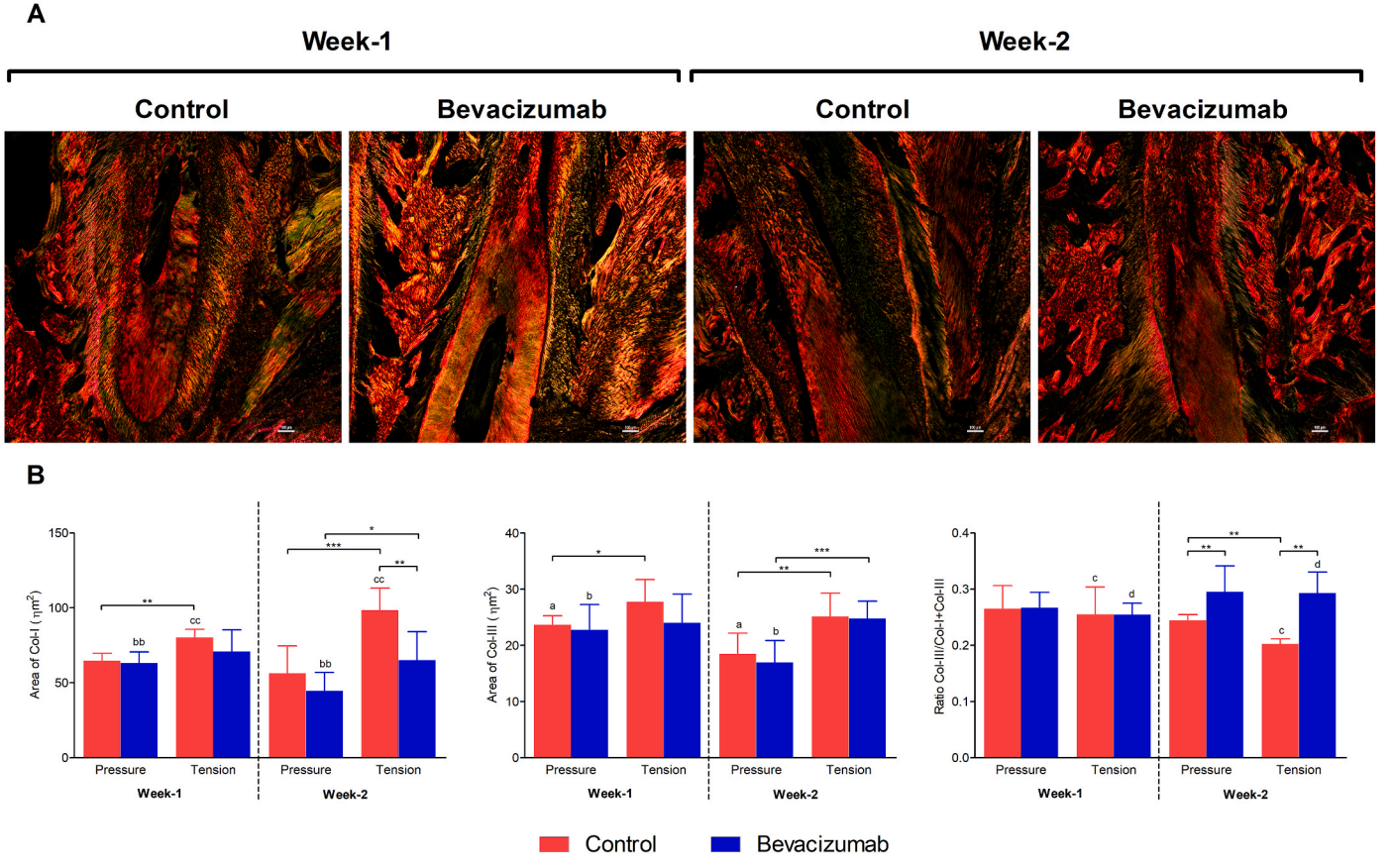

Figure-5. Evaluation of collagen fibers expressions at pressure and tension sites. (A) Microphotography of picrosirius red staining after 1 and 2 weeks of orthodontic force. Mature collagen-I fibers are in red (Col-I), while immature collagen-III fibers are in green (Col-III). Slides were inspected under magnification of 40× (scale bar = 100 μm). (B) Semi-quantitative measurement of Col-I and Col-III fibers. Numerical data is presented as Mean ± SD (n = 6) and significance is presented as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Similar small letters indicate significance ($p < 0.05$ and $p < 0.01$) between time points within the same group and site. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

timepoint ($p < 0.05$) **(Figure-3 B)**. TRAP staining showed various osteoclasts presentations particularly at the pressure site **(Figure-4 A)**. After 1 and 2 weeks of OTM, there were more $TRAP^{+}$ stained cells ($p < 0.01$) at the pressure side in the control and bevacizumab groups as compared to the tension sites. The pressure site showed a remarkably ($p < 0.05$) higher proportion of $TRAP^{+}$ cells in the bevacizumab group *vs.* control **(Figure-4 B)**.

Picrosirius red staining showed that the mature Col-I was the more predominant type than the immature Col-III at the pressure and tension sites at the two time points **(Figure-5 A)**. The color histogram analysis revealed that the Col-I area was significantly ($p < 0.01$) higher in control group compared to bevacizumab group at the tension site, after 2 weeks. When comparing the sites, both Col-I and Col-III were higher in the tension sites than pressure sites in the control group, after 1 week ($p < 0.01$) and 2 weeks ($p < 0.001$) of OTM. In contrast, significantly higher Col-I and Col-III areas in the tension *vs.* pressure sites in the bevacizumab group were only observed after 2 weeks ($p < 0.05$) of orthodontic force. The ratio of immature Col-III area to total collage area was on par for the bevacizumab and control groups after 1 week. However, after 2 weeks, significantly higher Col-III ratios were found in the bevacizumab group than the control, both in the tension and in the pressure sites **(Figure-5 B)**.

4. Discussion

The present study explored whether the antiangiogenic therapy by bevacizumab in the dose of 10 mg/kg twice per week, could impact OTM in rat model. This dose of bevacizumab was selected based on previous animal investigation, with which no major adverse effects were observed, as well as being also comparable to human dose (7.5–10 mg/kg IV) [13]. The choice of intraperitoneal route instead of intravenous was mainly due to its ease of use and to minimize the stress to animals, avoiding the need for anesthesia during the consecutive administrations. It is also worth mentioning that bevacizumab is a monoclonal antibody, which will be subjected to degradation upon oral administration, and hence the oral route was excluded. In addition, due to the less background knowledge on the impact of local bevacizumab administration, we preferred to firstly prove our hypothesis using the systemic administration route that is the most documented approach in which bevacizumab induce its potent anti-vascular effects.

In line with the literature, OTM model generated two opposing sites characterized by different bone formation and bone resorption activities, i.e., the pressure and tension sites revealed enhanced osteoclastic bone resorption and osteoblastic bone formation activities, respectively. Bevacizumab boosted tooth movement and affected the alveolar bone microstructure at both sites. These changes were associated with pronounced reduction in the osteoblastic bone formation, and enhanced osteoclastogenesis. Additionally, the distribution of collagen motifs, whether type I or III, was dysregulated by bevacizumab therapy at both tension and pressure sites.

Our study reports that bevacizumab influences tooth movement in response to orthodontic force application. Macroscopic evaluation showed that bevacizumab effect on OTM was more prominent after 2 weeks. In the present study, the orthodontic force was applied in a continuous manner. Continuous application of orthodontic force produces a faster movement and may be linked to a higher root resorption risk compared to intermittent force [17]. Importantly, the observed bevacizumab-accelerated OTM did not appear to induce an evident root resorption. Moreover, the micro-CT data revealed that bevacizumab reduced bone volume, trabecular number and thickness in both pressure and tension sites. On the other hand, bevacizumab increased trabecular separation only in the pressure site, while reducing it in the tension site. The microstructural findings are, at least, partly, and indirectly supported by previous studies showing that bone density and blood vessels presentation are boosted when osteoblasts are genetically-modified to overexpress VEGF [4]. Therefore, it could be speculated that suppression of VEGF by bevacizumab hinders the bone formation response at the tension site, possibly *via* decoupling between angiogenesis and osteogenesis. In contrast, it is not evident yet why bevacizumab triggered higher bone resorption at the pressure site. A pleasurable explanation is that the inhibited osteogenic processes had affected the regulatory mechanisms typically exerted by osteoblasts on osteoclasts. This assumption is supported by the previous study where VEGF overexpression although promoted bone formation and bone mass, it resulted in a dramatic reduction and almost absence of $TRAP^{+}$ osteoclasts, by day 14, in specific regions near the sites of promoted angiogenesis and osteogenesis in mice fracture healing model [4]. The authors in the later study found a significant increase in immunoreactivity of OPG, a major inhibitor of osteoclastogenesis, in the regions of promoted angiogenesis/osteogenesis [4]. Collectively, the results suggest that the bevacizumab-induced inhibition of VEGF firstly reduces the osteogenesis, due to decoupling from angiogenesis, and this subsequently leads to enhanced osteoclastic activity, likely due to a reduction in osteoblast regulatory mechanisms.

The present findings show that the net effect of reduced bone formation at the tension site and enhanced bone resorption at the pressure site could explain the enhanced tooth movement by bevacizumab. The reported reduced bone formation by bevacizumab at the tension site agrees with multiple previous studies, which highlight the vital role VEGF on osteoblastic activity. Downregulated VEGF expression induced apoptosis of osteoblasts through PI3K/AKT signaling pathway [18]. Moreover, the osteoblastic expression and production of VEGF are increased during bone fracture healing [19]. Nonetheless, our results were not in agreement with Kohno et al. study, where the administration of polyclonal antibodies against VEGF resulted in a fewer number of osteoclasts in conjunction with suppressed tooth movement [20]. The reason for these discrepancies could be related to the different doses and experimental durations between the two studies. Furthermore, the present results are not in harmony with earlier studies which reported that over-expression of VEGF might lead to an enhanced osteoclast recruitment and paradoxical bone loss [21]. Given the well documented stimulatory role of VEGF on osteoclasts, the exact reason for the demonstrated enhancement of osteoclastic resorption, in the pressure site, by interfering with VEGF, is yet to be determined. Another plausible explanation might be related to the involvement of osteocytes. Osteocyte is a well-known regulator for bone remodeling, and they express and secrete major osteoclast promoting signals, including RANKL, during bone remodeling processes [22–26]. Moreover, it has been shown that osteocytes respond to compression force by increasing the expression and secretion of RANKL and VEGF both in vitro and in vivo, in rat OTM model [27]. To our knowledge, there is no studies which prove the expression of VEGF receptors by osteocytes, neither any data on inhibitory effects of

anti-vascular medications on osteocytes. Therefore, we can postulate that RANKL and possibly VEGF released by "unaffected" osteocytes may have safeguarded the osteoclastic activity, especially in the pressure side. We can also speculate that RANKL expressed by osteocytes may have further dysbalanced the RANKL/OPG ratio, leading to a net effect of a high catabolic activity, particularly in the pressure side. Nonetheless, such hypothesis requires detailed mechanistic study to determine the site-specific expression and secretion of the major bone remodeling factors after administration of anti-vascular medication in the OTM model.

The histological evaluation shows that bevacizumab reduces the relative proportion of osteoblasts, particularly at the tension side. On the opposite pressure side, bevacizumab showed an enhanced bone resorptive activity with exaggerated proportion of osteoclasts and restricted vascularization. Overall, the current study supports previous research assuming that compromised vascular supply would negatively impact the bone response to trauma, inflammation, and, possibly, mechanical force through inhibition of osteoblastic bone formation [28]. Orthodontic force inevitably results in inflammatory environment necessary for bone resorption that facilitate the tooth movement [17]. Studies has revealed that OTM enhances biological signals, such as interleukine-1β (IL-1β), which imply biological inflammatory changes that might lead to apical root resorption [29]. Although some studies reported that osteoclasts differentiation, which is augmented by inflammation, is reduced following exposure to *anti*-VEGF antibodies [30], bevacizumab, in the present study, increased the $TRAP^{+}$ osteoclast at pressure side. Taken together, it could be speculated that osteoclasts response to bevacizumab therapy is diverse, which may involve both direct osteoclast inhibition, *via* VEGF receptors on osteoclasts, and/or osteoclast stimulation, *via* the inhibition of osteoblast regulatory mechanisms.

Bevacizumab impact on OTM can be further explained by the differential spatial distribution of different collagen types, which play major roles in force transmission, and overall bone biomechanics [31]. The orthodontic force is linked to collagen remodeling [32]. Studies have found that orthodontic mechanical load might enhance the expression of Col-III, especially at the tension site, whereas the expression of Col-I fibers would be decreased by orthodontic force [33]. It could be that the enhancement of Col-III deposition will relieve the tension force and, hence, increase the Col-III/Col-I ratio particularly at the early phase of OTM. Furthermore, Col-III/Col-I ratio will be adjusted at the late phase of OTM by more accumulation of the mature Col-I. In this study, both collagen types had higher proportion at the tension sites in contrast to the pressure, irrespective of bevacizumab treatment. Interestingly, bevacizumab markedly reduced the Col-I expression in the tension site and potently augmented the Col-III/Col-1 ratio in the tension and pressure sites, after 2 weeks of OTM. In agreement with our study, inhibition of VEGF by bevacizumab downregulated the expression of Col-I in primary human alveolar osteoblasts, which was postulated by the authors of the previous study as a potential explanation for bevacizumab-associated MRONJ [28]. Moreover, studies showed that enhanced angiogenesis is associated with higher collagen production, especially the type I collagen (Col-I) [34]. Accordingly, restricted angiogenesis can be anticipated as a plausible cause for the reduced Col-I production observed in the present study.

The present study is the first in vivo experiment that assesses the impact of the anti-vascular therapy, using bevacizumab, on alveolar bones response to orthodontic mechanical force. As another strength, this study provided evidence that bevacizumab effects on bone remodeling are site specific and influenced by the type of mechanical force (whether it is tension or pressure). On the other hand, one limitation of this study is the extrapolation of the present animal data to the human clinical setting. Although well-documented, the present OTM model does not totally resemble the clinical settings. Another limitation is the semi-quantitative analysis of the histology and $TRAP^{+}$ cells. Standardization of the histological sections, and consequently the images, represented a difficulty despite the efforts made to cut multiple similar sections. Additionally, evaluation of molecular mechanisms associated with bevacizumab effects would have provided further explanations of the observed effects.

Eventually, testing the local effects of bevacizumab on OTM would be an interesting prospective experimental and/or therapeutic direction. The local application of bevacizumab has been recently documented to treat pathological conditions, such as the age-related macular degeneration [35]. As a new direction, the concept of local administration of bevacizumab to accelerate OTM may provide promising strategy to improve orthodontic therapies especially for the complicated cases. However, more studies are needed to determine the best local bevacizumab administration method in conjunction with OTM.

5. Conclusions

Our findings suggest that the antiangiogenic therapy by bevacizumab accentuates the tooth movement under orthodontic loading *via* alteration of microarchitecture at pressure and tension sides during the initial OTM phase. VEGF restriction due to continuous bevacizumab administration seems to diminish osteoblastic bone formation, at the tension side, disbalancing it with the osteoclasts bone resorption at the pressure side. Continuous bevacizumab infusion and the associated alterations in bone remodeling at both sides also align with less proportion the mature collagen fibers, which may further facilitate tooth movement. This study provided pre-clinical evidence that VEGF inhibition would alter alveolar bone reaction to orthodontic force. Future translational clinical studies are warranted, which may result in modified orthodontic therapy in patients on antiangiogenic bevacizumab medications.

Ethics statement: The study was approved by the institutional review board at Imam Abdulrahman Bin Faisal University (IRB# 2020-02-301).

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Author contribution statement

Hatem Abuohashish: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Abdulaziz Alamri; Suliman Shahin; Dalal Almazrou; Taleb Alkhamis: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Omar Omar: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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