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Analysis of microangiogenic patterns at differently and constantly loaded implants in the rat tail model – a micro-computed study

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Zusammenfassung

Mini-Implantate generieren ein immer größeres Interesse unter Zahnärzten, da sie ungewollten Zahnbewegungen während der kieferorthopädischen Behandlung vorbeugen. Sie gelten als temporäre, ortstabile skelettale Verankerung. Jüngste Studien haben jedoch gezeigt, dass Mini-Implantate innerhalb des Knochens wandern können, ohne an Stabilität zu verlieren, wenn sie einer kontinuierlichen, niedrigen Kraft ausgesetzt sind. Dies wird als Implantatmigration bezeichnet. Es wird vermutet, dass diese Implantatmigration mit einem Knochenumbau einhergeht. Dies setzt wiederum die Bildung neuer Blutgefäße voraus. Ziel der folgenden Studie war es daher, die mikroangiogenen Muster um wandernde Mini-Implantate zu untersuchen.

Dazu wurden bei 16 Ratten zwei speziell angefertigte Mini-Implantate in einem Schwanzwirbel inseriert und mit Kontraktionsfedern verbunden (Kräfte: 0 N, 0,5 N, 1,0 N, 1,5 N). Nach einer zwei- bzw. achtwöchigen Belastung wurden die Tiere vor und nach der Gefäßperfusion mit dem Silikonkautschuk mittels eines Micro-Computertomographen gescannt. Die Gefäße wurden durch Subtraktion der beiden konsekutiven Scans segmentiert. Es wurden die Gefäßdicke (V.Th.), das Gefäßvolumen pro Gesamtvolumen (VV/TV) und die Gefäßabstände (V.Sp.) innerhalb eines periimplantären zylinderförmigen Volumens um jedes Implantat erfasst. Dieses Volumen wurde zudem in zwei laterale, einen proximalen (in Kraftrichtung) und einen distalen (entgegen des Kraftvektors) Quadranten unterteilt. Zudem wurde der Einfluss der Metallartefakte auf die Erhebung der Gefäßparameter untersucht. Für die statistische Analyse wurden linear gemischter Modelle angewendet, die mithilfe der Software R durchgeführt wurden.

Die Kraftgröße korrelierte positiv mit den VV/TV- und V.Th-Werten nach zweiwöchiger Belastung. Nach achtwöchiger Belastung wurden keine derartigen signifikanten Unterschiede festgestellt. Außerdem waren nach zwei Wochen VV/TV im proximalen und die V.Sp. Werte im distalen Quadranten signifikant erhöht, während nach acht Wochen die VV/TV Werte im distalen Quadranten signifikant höher war.

Summary

Orthodontic mini-implants are gaining interest in the dental field as they can prevent unwanted tooth movements during orthodontic treatments. They are supposed to provide temporary, stationary skeletal anchorage. However, recent studies indicated that the mini-implants may migrate in bone without losing stability when subjected to a continuous force of low magnitude. This has been referred to as implant migration. It is believed that this migration is accompanied by bone remodeling. This in turn would require the formation of new blood vessels. Therefore, the following work aimed to assess microangiogenic patterns around mini-implants migrating in bone.

For this purpose, two customized mini-implants were inserted into one caudal vertebra from 16 rats and connected with a contraction spring (forces: 0 N, 0.5 N, 1.0 N, 1.5 N). After two and eight weeks of loading, the animals were scanned using micro-computed tomography before and after vascular perfusion with the silicone rubber. Vessels were segmented by subtracting the consecutive scans. Vessel thickness (V.Th.), vessel volume per total volume (VV/TV), and vascular spacing (V.Sp.) within a peri-implant cylindrical volume of interest (VOI) around each implant were recorded. This VOI was further divided into two lateral, one proximal (in the direction of the force), and one distal (against the direction of the force vector) sector. In addition, the influence of metal artifacts on the collection of vascular parameters was examined. For the statistical analysis, linear mixed models were performed using the software program R.

Force magnitude positively correlated with VV/TV and V.Th values after two weeks of loading, whereas no significant associations were found after eight weeks of loading. Furthermore, after two weeks, VV/TV significantly increased in the proximal, while V.Sp. significantly increased in the distal sector. After eight weeks of loading, VV/TV values were significantly higher in the distal sector.

List of Abbreviations

2W	two-week
3D	three-dimensional
8W	eight-week
BMP	bone morphogenic protein
CD	Cluster of Differentiation
FGF	fibroblast growth factor
IL	interleukin
kV	kilovolt
M-CSF	macrophage colony-stimulating factor
MMP	Matrix metalloproteinase
N	Newton
OPG	osteoprotegerin
PDGF	platelet-derived growth factor
PMN	polymorphonuclear neutrophil
RANK	Receptor Activator of NF-κB
RANKL	Receptor Activator of NF-κB Ligand
RUNX2	Runt-related transcription factor 2
TGF	transforming growth factor
TNF	tumor necrosis factor
V.Sp.	vessel spacing
V.Th.	vessel thickness
VEGF	vascular endothelial growth factor
VOI	volume of interest

VV/TV	vessel volume per total volume
Wnt	portmanteau of the int-1 and the Wg (Wingless) gene
µCT	micro-computed tomography

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1 Introduction

In recent decades, mini-implants have emerged as a powerful device in orthodontics to control tooth movement in high-anchorage demanding cases without unintentional dentoalveolar effects [1, 2].

Screwed into the upper or lower jaw of the patient, they have been believed to provide absolute anchorage during tooth movement. However, recent findings suggested that mini-implants can display intraosseous movement when subjected to a constant force over a period of time [3-7]. As implants do not possess the periodontium that usually initiates the intraosseous movement from teeth [8, 9], the mechanism of this so-called implant migration is yet to be understood. However, it is believed to be caused by the remodeling of surrounding bone due to the applied force.

As this remodeling is dependent on precursor cells, growth factors, oxygen, and various nutrients for bone mineralization, a change in the vasculature's morphology for a sufficient blood flow is inevitable [10, 11]. Compromised angiogenic activity in bone and the subsequent lack of blood supply have been associated with numerous skeletal diseases, i.e., osteonecrosis and osteoporosis [12-17].

Angiogenesis can be analyzed by using either histochemistry or software-based micro-computed tomography (μCT). A disadvantage of histological slices is that it is difficult and time-consuming to procure three-dimensional (3D) data by reconstruction [18]. Thus, an approach using μCT was aimed to be used in this study to fully evaluate the spatial distribution of vessels around the implants.

Because greyscale values of the contrast agent fall into the same range as that of bone, bone tissue is often decalcified before scanning to enable the assessment of perfused vessels [19-21]. However, the present study intended to analyze the intraosseous angiogenic patterns by subtracting pre-contrast scans from post-contrast scans, and by analyzing them in the direct vicinity of differently loaded mini-implants, which were inserted into caudal rat vertebrae.

1.1 Mini-implants

Mini-implants can be used for a variety of indications during orthodontic treatment. They present an effective skeletal anchorage that proves to be helpful in malocclusion cases, where traditional orthodontic treatments may otherwise encounter difficulty, e.g., edentulous patients [22], as well as extraction space closures [23].

Anchorage, conventionally achieved by using the patient's teeth, can be defined as the resistance offered when subjected to a force [24]. Ideally, anchorage systems should remain stationary and record no anchorage loss.

Skeletal anchorage that, otherwise, can only be found in ankylosed teeth, offers a reduction in dentoalveolar effects while at the same time maximizing the orthopedic impact in growing patients with equivalent if not superior results [25, 26]. Thus, mini-implants are encountered with ever-increasing popularity amongst orthodontists [27-29].

Clinical Applications

Since their initial reference in 1945, when Vitallium miniscrews were first used for teeth movements in an *in vivo* experiment with dogs [30], there have been continuous efforts to develop an orthodontic absolute anchorage system through mini-implants.

Mini-implants can be deployed in all three planes of space: anteroposterior, transverse, and vertical [26, 27, 31]. This includes the uprighting of molars [32-34], protraction of posterior teeth [35], the correction of an open bite by means of intruding maxillary molars, and en-masse retraction of the maxillary front, as well as distalization of molars to correct Angle class II malocclusion [25, 36-45] in growing and adult patients alike [46].

Additionally, mini-implants pose as an alternative treatment for mild cases of Angle class III malocclusion, either individually or in combination with other alternative therapy methods, e.g., orthodontic facemasks, to promote maxillary growth [28, 47, 48]. Studies also suggested that mini-implants can serve as an

alternative method for the intrusion of maxillary incisors in deep bite patients with a similar to slightly more favorable outcome than intrusion arches [49, 50].

Loading Time and Insertion Locations

Since orthodontic mini-implants do not require osseointegration, they can be loaded immediately after insertion [51], which is still controversially discussed for dental implants. The duration of the loading does not influence the failure rate of mini-implants, as one study has reported. Nonetheless, it has to be noted that implant stability usually decreases significantly until the fourth week after insertion owing to peri-implant remodeling and the loss of primary stability [52].

Generally, orthodontic mini-implants are inserted into the hard palate, the chin, edentulous areas, or in the interradicular spaces between tooth roots, either buccally or orally [53]. In the maxilla, mini-implants are often deployed in the midpalatal suture region or paramedian, as the bone in this area tends to exhibit a dense cortical layer and keratinized gingiva [54, 55]. For the mandible, the most common insertion locations are the interradicular spaces between the posterior portion of the arch, usually between the second premolar and first molar, or between the first and second molar, attributable to the sufficing bone thickness [53, 56].

In the past, orthodontic mini-implants have been placed with a flap surgery, although in recent years, it has been shown that patients experience less pain and discomfort if the mini-implant is inserted without flap surgery [57, 58].

Implant Properties

Mini-implants are predominantly made out of grade V titanium (Ti6Al4V), as its mechanical properties are superior for orthodontic anchorage compared to pure titanium [51], and are also biocompatible at a physiological pH of 7 [59, 60]. Typically, a conical thread design is preferred to a cylindrical design because the former achieves higher primary stability [61].

Mini-implants used during orthodontic treatment may vary in diameter, depending on insertion site and loading force to ensure primary stability [62-65]. For insertion in an interradicular space, a smaller diameter is favored to minimize the risk of root damage. However, mini-implants with a smaller diameter may be more prone to displacement than their wider counterparts [66]. Generally, a diameter smaller than or equal to 1.0 mm has shown to have a significantly lower success rate [56, 58].

To determine the length, the orthodontist must gauge the quality and quantity of the bone as well as the location of the insertion site and the intended loading force to provide primary stability [67, 68]. Implant stability has been shown to vary significantly according to the insertion depth and implant length [63, 65, 69, 70]. In areas with thick cortical bone, mini-implants of shorter length are preferred, whereas longer mini-implants provide stability in trabecular bone [70]. Especially for clinical situations that demand high anchorage, bicortical insertion with increased implant length may be more suitable [71], although the use of long mini-implants is related to increased risk of tissue damage and placement complications [72]. For the palatal region, mini-implants of greater lengths are recommended as well, as they have been shown to provide higher primary stability [73].

Benefits of Using Mini-implants

Apart from the effectiveness of using mini-implants as skeletal anchorage in orthodontic treatment, a crucial advantage is that they can be used without special patient cooperation, as compliance is a critical factor regarding the success of orthodontic treatment [74]. After insertion of the screws, no other strict instructions must be followed by the patients as opposed to conventional methods, e.g., the daily wearing of removable appliances or adjuncts [75].

Another advantage is their ability to be immediately loaded [76], as well as improved aesthetics when compared to other traditional methods, such as headgears [77]. Moreover, mini-implants have displayed a favorable soft tissue profile when compared to conventional anchorage devices [78].

1.2 Bone Remodeling

Bone remodeling describes a lifelong process wherein bone resorption and bone formation are tightly coupled [79]. Annually, around 10% of bone is replaced [12]. It is, amongst others, influenced by hormonal levels, inflammation, and lack of mechanical stimulation, however, it is not solely damage-driven [80]. Bone remodeling is not only regulated by the traditional bone cells – osteoclasts, osteoblasts, and osteocytes – but also innate and adaptive immune cells, such as polymorphonuclear neutrophils (PMN), B and T cells. The process is modulated by mediators such as interleukins, leukotrienes, chemokines, prostaglandins, bone morphogenic proteins (BMP), and Wnt signaling pathways [81].

Several diseases are linked to a disrupted homeostasis of bone remodeling, such as arthritis, proneness for bone fractures in diabetes mellitus patients, post-menopausal osteoporosis, and periodontal disease [82-86]. Bone remodeling also plays an important part in the osseointegration of dental implants and the orthodontic movement of teeth [8, 9, 87, 88].

Types of Bone Cells

Osteoclasts are found in resorption bays, also called Howship's Lacunae, and are adapted to remove mineralized bone matrix [81, 89]. These large terminally differentiated myeloid cells dock to the bone surface through RGD-binding sites, a tripeptide consisting of Arginine, Glycine, and Aspartate. There they produce acid that dissolves mineral content, and enzymes such as matrix metalloproteinases (MMPs), which remove the organic matrix consisting of collagen, elastin, and laminins [90]. As osteoclasts form by two or more precursor cells fusing together, they usually have numerous nuclei.

Osteoblasts form new bone during the remodeling process. They stem from osteoprogenitor cells and have only one nucleus. Their differentiation is controlled by the Runt-related transcription factor 2 **(**RUNX2) and other transcription factors [91]. During their terminal differentiation, subpopulations of osteoblasts engulf themselves by osteoid, which they produce themselves.

Osteoid describes the organic portion of bone that is not yet mineralized, mainly consisting of type I collagen [92]. These cells are then referred to as osteoid osteocytes.

With approximately 95%, osteocytes are the most numerous cell type found in mature bone and reside in lacunae [90]. These long-lived cells have dendritic processes with which they can communicate with other osteocytes and bone lining cells [93, 94]. Bone lining cells are osteoblasts that do not differentiate from osteocytes or undergo apoptosis [95]. They promote the differentiation of hematopoietic stem cells into osteoclasts through their communication with osteocytes [94, 96]. Osteocytes do not only react to mechanical but also metabolic signals, e.g., a decrease in estrogen and aging result in an increased rate of osteocyte death [93, 97].

Sensible to mechanical load, these osteocytes express sclerostin. Sclerostin inhibits Wnt signaling pathways, which in turn directs bone formation. However, they also secrete the transforming growth factor β (TGF-β) that inhibits osteoclastogenesis, i.e., the resorption of bone through osteoclast differentiation [98]. When mechanically stimulated, they produce osteoclastogenic factors, e.g., receptor activator of the NF-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) to promote bone remodeling [82].

Mediators of Bone Resorption and Formation

RANKL and M-CSF are dominant pathways that upregulate osteoclast formation and activity [99, 100]. Depending on the etiology, e.g., inflammatory response, post-menopausal osteoporosis, etc., RANKL can be secreted by immune cells, such as B and T cells and monocytes, or osteocytes, osteoblast precursor cells, and marrow stroma cells [82, 99]. In physiological bone remodeling, during which the bone volume does not change [101], osteocytes are the cells that predominantly express RANKL, as well as its inhibitor osteoprotegerin (OPG).

RANKL binds to the receptor activator of NF-κB (RANK), which is located on the surface of osteoclast precursor cells, which activates NF-κB and other signaling

pathways [102]. This promotes the formation, activation, and the survival of osteoclasts and, therefore, promotes bone resorption [103].

M-CSF, on the other hand, is a glycoprotein growth factor that modulates the differentiation, proliferation, and survival of monocyte/macrophage lineage cells. It is expressed by osteoblasts and bone marrow progenitor cells and activates an intracellular cascade in osteoclast precursor cells. This cascade leads to the latter's proliferation.

OPG belongs to the tumor necrosis factor (TNF) family and is released by osteoblasts and fibroblasts, amongst others. It is a natural inhibitor of RANKL, as it binds to it, thus inhibiting RANKL from binding to its respective receptor, RANK [104]. Therefore, the expression ratio of RANKL and OPG affects the degree to which osteoclasts are formed and active [105].

A suppression of physiologic bone resorption due to downregulation of RANKL and upregulation of OPG is also accomplished by Wnt signaling pathways. The name Wnt is a portmanteau of the int-1 (mouse locus for the mouse mammary tumor virus) and the Wg (Drosophila melanogaster *wingless*) gene [106]. Wnts are secreted glycoproteins that, in addition to a suppression of osteoclastogenesis, play an important role in stimulating bone formation through osteoblast differentiation, embryonic development, and tumorigenesis [107, 108]. There is one β-catenin dependent canonical Wnt pathway and three non-canonical Wnt pathways, which are β-catenin independent [81]. It is the former that in osteocytes is required for normal bone homeostasis [107, 109].

As previously mentioned, sclerostin is a Wnt inhibitor that is also produced by mature osteocytes. It binds to the pathway, thus inhibiting the proliferation and differentiation of osteoblasts and increasing their apoptosis [110]. Moreover, sclerostin induces osteocytic osteolysis [109].

Other important mediators include BMPs and fibroblast growth factor-2 (FGF-2). BMPs play a vital role in skin formation and hair follicle development [111] but also stimulate bone formation by activating the RUNX2 gene that controls the differentiation of osteoblasts [98]. FGF-2 is expressed in osteoblasts and mesenchymal cells and is involved in a variety of cellular processes such as angiogenesis, wound healing, limb formation, tumorigenesis, and bone biology

[112]. As a bone anabolic stimulant, FGF-2 regulates the canonical Wnt signaling pathway and the activation of RUNX2, therefore promoting osteoblast differentiation, although it has also been found to have a secondary stimulatory effect on osteoclastogenesis [113].

Immune Cells During Bone Remodeling

Among the innate immune cells, PMNs, dendritic cells, and monocytes/ macrophages are the predominant cells that modulate bone resorption, especially under inflammatory conditions.

PMNs outweigh during the initial acute inflammatory phase and are recruited by chemokines from the peripheral vasculature. They express RANKL, as well as many inflammatory cytokines, such as IL-1β, TNF-α, IL-6, and more [82, 114]. Monocytes differentiate from macrophages in tissue and can have M1 or M2 phenotypes. M1 macrophages produce pro-inflammatory cytokines like IL-1β, TNF-α, as well as RANKL, whereas M2 macrophages act as anti-inflammatory cells, secreting mediators such as IL-4 and IL-10 [114, 115]. Dendritic cells function as antigen-presenting cells and therefore regulate the homeostasis of the immune response [116]. They travel to the lymph nodes to activate lymphocytes.

Lymphocytes, such as B and T cells, are part of the adaptive immune system. Both can express RANKL and various other cytokines when activated to promote osteoclastogenesis. T cells can develop into T helper cells, Th1, Th2, and Th17 cells, of which Th1 and Th17 cells express pro-inflammatory cytokines, such as IL-1 and TNF-α, as well as RANKL, thus promoting osteoclastogenesis [117, 118], while Th2 cells produce anti-inflammatory cytokines like IL-4 and IL-10 and therefore downregulate osteoclastogenesis and bone loss [119].

Pathologies of Bone Remodeling

Many diseases of the bone can be traced back to disruptions in the physiological process of bone remodeling, either due to inflammation, hormonal changes, or lack of mechanical stimulation. In osteoporosis, by far the most prevalent disorder

of bone remodeling, bone resorption outweighs bone formation during the remodeling process. Nonetheless, disorders in bone remodeling can have different etiologies and therefore classifications, e.g., primary (post-menopausal or age-related) or secondary (glucocorticoid or immobilization induced) osteoporosis, Paget's disease, osteopetrosis, etc. [79, 118].

Diabetes mellitus, a chronic metabolic disease, results in hyperglycemia, higher levels of advanced glycation end-products, and reactive oxygen species. This leads to a heightened expression of inflammatory cytokines, increasing numbers of osteoclasts, and reducing osteoblasts and bone formation [86, 98, 120-123].

Autoimmune diseases can also influence bone remodeling. Around 0.5-1.0% of the western population suffers from Rheumatoid Arthritis [83], which stems from a massive influx of monocyte/macrophage precursors into the diseased synovium [84] and their maturation into osteoclasts, helped by the abundance of M-CSF and RANKL in the rheumatoid synovial membrane [85].

Periodontitis has been associated with an upregulation of RANKL and a downregulation of OPG, thus increasing the RANKL/OPG ratio and the resulting bone resorption [105]. A recent study suggested that osteolysis of the alveolar bone may be a means to remove the cause of the inflammation by provoking tooth loss [124]. The excessive release of the cytokine TNFα by macrophages may lead to bone loss during inflammation due to Porphyromonas gingivalis, a bacterium strongly implicated in periodontitis [125]. Moreover, the production of RANKL by osteocytes plays a critical role during the inflammation-based bone loss in periodontitis patients [126].

The Role of Bone Remodeling in Dental Practice

Bone remodeling holds a critical role in the usage of dental implants to replace lost teeth in patients. Osseointegration, in which bone remodeling plays part in, is one of the most important factors regarding the clinical success of dental implants [87, 88]. After the initial inflammation in response to the trauma due to the implant placement, peri-implant osteogenesis provides active biological fixation of the implant [127]. Afterwards, the phase of bone remodeling begins,

wherein osteoclasts replace woven bone with lamellar, more mineralized bone directly onto the implant surface, which provides a more stable secondary fixation of the implant [128].

In recent years, however, it has been noted that it is not only teeth that can move in bone without losing their stability but also mini-implants, which were thought to be an absolute anchorage for orthodontic treatment [3, 4, 6, 7]. Different studies belonging to the same project this dissertation is part of, have found that an increased bone apposition was observed in animals in which the mini-implants were subjected to a higher degree of force and showed a higher rate of implant migration [5, 129].

1.3 Angiogenesis

Except for cartilage and the cornea, which are avascular, blood vessels provide efficient distribution of blood supply throughout the body, as well as low diffusion distances from capillaries to the target tissue [130]. As not only oxygen but also necessary nutrients, cytokines, hormones, growth factors, neurotransmitters, etc., are transported via blood, the maintenance of the vascular network is of vital importance [10, 131]. The vascular system also regulates the body temperature and systemic pH and mediates immune response [132].

As opposed to vasculogenesis, which is defined as the blood vessel formation in the embryo, angiogenesis describes the remodeling and expansion of a pre-existing vascular network [133]. It is a highly complex process, both during homeostasis and when disturbances arise and is tightly regulated at a molecular and genetic level [134]. Angiogenesis continuously takes place in a functional adult vascular system according to tissue demands [135], e.g., during wound healing, the menstrual cycle, and pregnancy.

Cells involved in the process of angiogenesis include, but are not limited to, endothelial cells in the intimal layer, pericytes and smooth muscle cells in the media layer, Cluster of Differentiation 34+ (CD34+) fibroblasts, and stroma cells in the adventitia of the existing vessel, as well as circulating inflammatory and

progenitor cells [133]. Especially the interaction between endothelial cells and pericytes will be further expanded upon later in this section.

The vascular endothelial growth factor (VEGF) presents the most dominant regulative factor of angiogenesis. Particularly VEGF-A stimulates both physiological and pathological angiogenesis by binding to the VEGF receptor-2 on the surface of endothelial cells [136, 137]. As such, the signaling of VEGF has been a prominent target for anti-angiogenic therapy in the context of cancer patient treatment, as pathological angiogenesis is a major factor in tumor growth and metastatic spread [136-139]. The platelet-derived growth factor (PDGF) is another pro-angiogenic factor that recruits pericytes and helps them mature to stabilize newly formed blood vessels [138, 140, 141].

Types of Angiogenesis

There have been several types of angiogenesis defined in literature, which can be broadly divided into sprouting, the more frequently studied form, wherein a new vessel sprouts out of a pre-existing vessel, and non-sprouting angiogenesis, e.g., intussusceptive angiogenesis, describing the splitting or remodeling of a pre-existing vessel by means of transcapillary pillars [135]. The latter is an important component of the remodeling process [142].

Although the groundwork of the vascular network is laid during vasculogenesis, sprouting plays a vital role during the later stages of organogenesis in the embryo as well [135, 143]. Sprouting angiogenesis is also part of a model termed “angioadaptation” [130]. In the past, it was generally considered that sprouting, remodeling, and pruning, are subsequent events. A remodeling of vessels hereby describes a change in vessel diameter, whereas pruning is defined as the loss of vessel segments. However, all three processes appear to be happening simultaneously, as recent findings have shown [130].

Mechanisms of Angiogenesis

For sprouting angiogenesis, angiogenic factors such as VEGF, angiopoietin-1, NOTCH, and various FGF subtypes stimulate the endothelial cells and pericytes

to release MMPs that degenerate the basement membrane [140, 144]. Inflammatory or hypoxic chemokines or those released by tumor cells can also induce angiogenesis [145].

Driven by angiopoietin-2, the detachment from endothelial cells and pericytes as well as the proteolytic degradation of the basement membrane results in the loosening of the endothelial cells. These cells, led by so-called endothelial tip cells with filopodia, which are activated by VEGF, follow a gradient of chemotactic factors into the extracellular matrix area, which is created by an increased permeability due to VEGF. There they form stalk cells, which, by cell proliferation, elongate the newly formed vascular sprout by trailing after the tip cells [145, 146].

Endothelial cells and pericytes stabilize this fragile new vessel by creating a new basement membrane [147]. Factors produced by pericytes, like TGF-β and angiopoietin-1 (an antagonist to angiopoietin-2), promote further vessel maturation and ensure the integrity of the brain-blood barrier [148-150].

Angiogenic Diseases

Angiogenic diseases are characterized by the abnormality of capillary growth as their main pathological feature [151]. These include psoriasis [152], infantile hemangioma [153], systemic sclerosis [154], arthritis [155], and diabetic retinopathy [132, 156], amongst others. Wound healing in patients with diabetes mellitus may also be impaired due to a deregulation of angiogenic factors, such as VEGF, PDGF, and other growth factors [157].

In addition, a lack of angiogenesis may result in skeletal diseases such as osteoporosis and osteonecrosis [13-15]. Osteogenesis and angiogenesis are tightly coupled processes [158-160]. Osteonecrosis, the death of bone tissue due to insufficient blood supply, occurs in the mandible as a result of radiation therapy, medication-induced, or after a fracture of the bone. Especially bisphosphonates used to prevent bone density loss in osteoporosis in some patients pose a risk for the so-called bisphosphonate-associated osteonecrosis of the jaw. Zoledronate, a commonly used bisphosphonate for breast cancer treatment, as it prevents bone metastasis, may inhibit vascular endothelial cell proliferation

directly, and even promote their apoptosis [161, 162]. In addition, it may reduce osteopontin levels, which has been associated with facilitating angiogenesis in bone [163]. Lastly, a recent review found that while osteonecrosis has been associated with a variety of biological therapies, the inhibition of angiogenesis has been the most common association [164].

Angiogenesis During Bone Remodeling

As opposed to most other tissues, bone can heal without forming fibrous scars. This might be due to the bone's unique ability to mimic the mechanics of fetal skeletogenesis during adult skeletal regeneration [165]. The timely appearance of blood vessels is hereby of critical importance, as the blood not only supplies the bone tissue with oxygen and nutrients but also with ions, namely calcium and phosphate, the building blocks for bone mineralization [146].

Periosteal, intraosseous, and periodontal ligament arteries provide the needed vasculature around teeth, whereas, for implants, the periodontal ligament arteries are not existent, therefore compromising the blood supply around them [12]. The periosteum is not only crucial for osseous growth, but also for bone regeneration, as it contains a large number of progenitor cells [12, 166].

VEGF and angiopoietin-2 expression increase initially in cutaneous wound healing until a new stable vascular network is formed [167]. The through hypoxia-activated hypoxia-inducible factor 1α appears to be the most prominent stimulant for the release of VEGF and, therefore, angiogenesis [158, 160].

Assessment of Angiogenic Patterns in Research

Although there are many ways to evaluate vasculature, including ultrasound and optical imaging, computed tomography (CT) is probably the most common method to assess angiogenic patterns. Histological examinations, while often used in the past, cannot easily provide a three-dimensional spatial distribution of vessels. *Ex vivo* imaging of angiogenic patterns can achieve higher resolution than *in vivo* analyses [21, 168], with up to 10 µm for *ex vivo* images compared to approximately 50-100 µm for *in vivo* images [169], although the maximum

resolution of novel scanners is in the range of 500 nm. However, *in vivo* CT imaging can provide non-invasive, longitudinal monitoring of the animal's vascular morphology [170, 171].

Nevertheless, the limitation in resolution and maximum scanning duration make the *in vivo* assessment of vessels difficult in small animals. Additionally, special precautions must be taken for the anesthesia and long-lasting dyes must be used, as to leave enough time for the scan [170].

1.4 Reference for Animal Testing

For this study, n = 16 female Albino rats of the Wistar strain were obtained from the Central Unit for Animal Research and Scientific Animal Welfare Affairs at Heinrich Heine University Düsseldorf. The study was approved under the reference number 84-02.04.2016.A380 by the local authority (Landesamt für Natur, Umwelt und Verbraucherschutz, Recklinghausen, Germany).

1.5 Aims of Thesis

This project was funded by the German Research Foundation (in German: *Deutsche Forschungsgemeinschaft*) and aimed to study different aspects of the migration of mini-implants in bone, including the rate of migration for differently loaded test groups [5], implant angles, bone remodeling, an analysis using the finite element method, as well as a histological examination and immunofluorescence analysis.

This dissertation aims to evaluate the microangiogenic patterns around mini-implants. While contrast-enhanced µCT imaging has been established as a reliable method to assess angiogenesis throughout the years, collecting the data of intraosseous vascular patterns remains demanding in both time and effort. Traditionally for *ex vivo* analyses, the bone needed to be decalcified after the

perfusion with the contrast agent, before it could be either scanned for a 3D reconstruction via µCT or histological slicing [21].

In this study, it was aimed to introduce a new method of assessing intraosseous vessels without the necessity of an additional step of decalcification of the bone.

Specifically, this study aimed to analyze:

1) whether there are differences in micro-angiogenetic patterns among the various test groups, i.e., high (1.5 N), medium (1.0 N) and low (0.5 N) loading groups, as well as the control group (0 N),

2) if the angiogenic activity differs between the proximal (in direction of the force vector) and distal (against the force vector) peri-implant sector,

3) if dissimilarities regarding angiogenic patterns exist between the early stages of healing, i.e., after two weeks (2W), and the late healing phase, i.e., after eight weeks (8W), and

4) whether metal artifacts influence the outcome.

To quantify the angiogenic activity, this study uses the indices vessel thickness (V.Th), vessel spacing (V.Sp.), and the vessel volume per total volume (VV/TV).

2. Micro-angiogenic patterns around orthodontic implants migrating in bone: A micro-CT study in the rat tail model, Hüfner, M., Rauch, N., Schwarz-Herzke, B., Knorr, I. J., Sager, M., Drescher, D., & Becker, K., Journal of Clinical Periodontology, 1– 10, (2021)

3. Discussion

Several clinical studies have observed displacement of orthodontic mini-implants in patients in the past [3, 6, 7, 172, 173]. However, the mechanism and factors facilitating implant migration are still unclear. In teeth, orthodontic tooth loading causes an aseptic inflammation due to hypoxia and fluid flow, thus promoting osteoclast resorption in compression zones, i.e., in the direction of the movement, and an increase of osteoblasts in areas of tensile zones [8, 9]. This ensures the continuing stability of the tooth during its intraosseous movement.

However, the conventional periodontal pressure-tension theory cannot be applied to mini-implants, as they do not possess a periodontium as a tooth does. Nonetheless, it has been postulated that a remodeling of the surrounding bone is responsible for implant migration [3, 4]. For this, angiogenesis may play a vital role, as in bone, blood vessels create niches for hematopoietic stem cells in the marrow [174, 175], as well as supply the bone tissue during growth, healing, and remodeling. Thus, this study aimed to assess whether angiogenic activity during implant migration differs according to the force applied, the peri-implant location, and the time of healing.

As a driving factor in angiogenesis, in bone, VEGF not only promotes vessel sprouting, which enables osteoblast precursor cells to reach the site of injury, it also stimulates endothelial cells to release osteogenic cytokines, such as BMP-2, to induce osteoblast differentiation directly [174, 176]. In addition, osteoblasts themselves release VEGF to further stimulate their activity, differentiation, and chemotaxis [159, 177-179]. Various pro-inflammatory cytokines, such as TNF-α, IL-1β, and other growth factors promote the expression of VEGF [155, 156, 180] and are released by immune cells, e.g., M1 macrophages, upon an aseptic inflammation during bone remodeling due to pressure or tension. Therefore, while a mechanical stimulus activates bone cells themselves, it also triggers the release of angiogenic growth factors, which in turn further upregulate bone remodeling directly through osteoblast differentiation. It might be this positive feedback loop that enables bone remodeling to allow implants to migrate without a periodontium.

This dependence on mechanical stimulus for implant migration is supported by the difference between test groups during the early healing phase. In 2W animals, higher loading groups had significantly higher levels of VV/TV and V.Th. than the low loading group and the control animals, which, in turn, correlates to the distance of the intraosseous implant movement, as previously published in the study by Becker et al. [5]. The decline of V.Th. in 8W animals might point to a short-term proliferation of vessels, as the vessel diameter, which is represented by V.Th., has been shown to be impacted by the release of VEGF. VEGF is released upon chronic local hypoxia to increase local endothelial cell proliferation and, subsequently, blood flow and O2 delivery [181].

Furthermore, VV/TV was elevated in the proximal sectors, i.e., in direction of the force vector, in 2W animals, whereas V.Sp. was significantly higher in the distal sectors. As aseptic inflammation during bone remodeling promotes the expression of VEGF, it is possible that angiogenic sprouting in the early healing phase is increased in compression zones rather than in tensile zones, regardless of the loading force. However, there was no difference between the two sectors regarding V.Th., neither in 2W nor 8W animals.

Moreover, in 8W animals, the level of VV/TV was higher in distal sectors when compared to proximal ones. This might indicate that in tensile zones, sprouting angiogenesis may be delayed when compared to compression zones. While there is no definite research on the matter, a recent study found that macrophage-induced angiogenesis through the release of VEGF in orthodontic tooth movement was significantly elevated in compression zones within 48 hours, while there has been no influence on the VEGF expression in tensile zones [182].

Furthermore, in the present study, some animals exhibited peri-implant bone defects. This was particularly the case for higher loading groups, which exhibited greater implant movement. These bone defects were by trend more often located or at least more pronounced in the distal sectors, i.e., tensile zones. This might be due to the direction of the implant migration, as the formation of osteoclasts, which are especially activated in the peri-implant compression zone, precedes the activation of osteoblasts in tensile zones following the initial stimulus in the remodeling cycle [183, 184]. However, it is also possible that these bone defects

occurred due to local osteonecrosis caused by a lack of blood supply. In any case, these defects may have played a role in the distal VV/TV levels as well. Moreover, when comparing the proximal sectors of 2W and 8W animals, 8W showed generally lower levels of VV/TV. This decline might be due to the pruning of vessels to prevent redundant branches obstructing efficient blood flow to tissues in need of supply [185].

After invasive dental procedures, e.g., the insertion of mini-implants, the periosteal microcirculation is importantly linked to wound healing [166]. It, therefore, stands to reason that an increased vessel density around the cortical layer of the bone is to be expected in this study, as sprouting angiogenesis is likely to start from bigger periosteal vessels. This was indeed observed in this study. However, no statistical analysis has been made because a further division into cranial and caudal sectors was difficult, as implants had different insertion depths, either due to the initial surgery or the implant migration.

Because mini-implants are subject to immediate loading, primary stability is crucial in terms of preventing implant failure. Primary stability in turn is dependent on implant design, and the soft tissue surrounding the insertion site, as well as bone quality, quantity, and density, especially that of the cortical bone [65, 70, 72, 186-190]. The cortical bone has shown a higher success rate with a thickness of at least 1 mm [190]. The rat vertebra has therefore been chosen for this study, as it encompasses both a cortical outer layer and an inner cancellous part that, in structure, resembles the human jaw.

Regarding the translatability to humans, the following aspects must be noted: At the moment there is a scarcity of knowledge regarding the age-related changes in bone remodeling in rat vertebrae. In humans, arch lengths and intercanine widths in humans change with age [191]. However, human jaws are considered to be among the most vital bones until old age, enabling implant placement in geriatric patients. Concerning angiogenesis, further research has to elucidate age-related changes in both rats and human jaws.

In a recent study, researchers found that while there was a significant decline between the amount of so-called type H vessels in the maxillae of infantile and juvenile mice, there was only little alteration between juvenile, adult, and aged

mice, respectively [192]. In contrast, it has been found that in other bones, such as the tibia and femur, there was a steady decline of type H vessels with age, which may link back to the fall of osteoblast numbers during aging [175]. Whether this translates to the physiology in humans, and, therefore, might explain the vitality of the human jaw bone into old age, remains to be investigated.

The results of further research following this study's design might, therefore, heavily rely on not only the age of the animals, even after the end of growth, but also the bone in which the data is analyzed. A relevant limitation of the rodent jaw is that it mainly consists of cortical bone, whereas human jaws are composed of both, cancellous and cortical layers.

A decreasing number of these type H vessels has been previously associated with a decline in progenitor cells for osteoblasts [193]. This decline is linked to an imbalance in favor of bone resorption which, in turn, is believed to be the reason for the deterioration of the bone and, therefore, a higher chance for pathological fractures in osteoporosis patients [13]. This recently discovered capillary subtype found in the murine bone metaphysis and periosteum may be responsible for coupling angiogenesis and osteogenesis [158, 174, 192]. A study showed that the majority of osteoblast and osteocyte progenitors, as well as RUNX2 and PDGF expressing cells, were found positioned in these vessels, even though type H endothelial cells make up only around 1.77% of all endothelial cells in the bone [158].

The researchers of the previously mentioned study also found a local increase of type H vessel numbers as well as RUNX2 in a molar extraction model in the alveolar bone of mice as early as 3 days post-surgery. This further indicates the subtype's importance regarding the coupling of angiogenesis and bone remodeling [192].

Unfortunately, these type H vessels cannot be identified through perfusion with contrast agent and µCT scanning. However, as these vessels possess particularly high levels of CD31 and Endomucin, they can be identified through immunostaining, as shown in the study by Yan et al. in 2020 [192]. To better understand the role of these vessels in bone remodeling during implant migration, further research should be conducted in the future.

Ex vivo µCT scans generate up to ten times higher resolution than *in vivo* imaging [21, 168]. Another disadvantage of *in vivo* dyes is their short time frame of enhancement. However, as they enable longitudinal monitoring [170, 171], they might represent the next step in understanding the forming of new angiogenic patterns in bone. Through this method of subtracting the pre-contrast scan from the post-contrast scan, longitudinal *in vivo* morphometry of intraosseous vessel may be possible. Thus, the role of vessel sprouting might be better understood in wound healing and various intraosseous diseases, like diabetes or tumors of the bone. Obstacles for *in vivo* analyses of the bone vasculature include the beforementioned limited time span of the contrast enhancement and the lower resolution due to the time frame set by the analgesia.

A limitation of this study were the metal artifacts caused by the nickel-titanium springs. As the implants were made out of grade V titanium, they caused very few metal artifacts. This is due to the higher atomic number of nickel when compared to the components of grade V titanium (titanium, aluminum, vanadium), which causes a higher than average amount of beam hardening [194]. Contrary to prior expectations, a standardized distance to the implant did not reduce the impact of streaking artifacts caused by the nickel-titanium springs. On the contrary, VOI's immediately surrounding the implants showed less variance when comparing the data from scans with and without implants in situ. As for why a standardized distance was not necessary, it is possible that the scatter radiation patterns canceled each other out, as they were present in both the pre- and post-contrast scans. However, it is yet unclear, why values dispersed more the farther away they were from the implant. The scatter radiation of pre- and post-contrast scans might overlap to a greater extent closer to their metal origin and vary more in shape and scale with distance to the implants.

As such a mutual negation of scatter radiation seems likely in this study, it is difficult to conclude whether the data regarding vessel parameters of the post-contrast scans without implants are more accurate than those with implants in situ, as the pre-contrast scans do always contain implants and, therefore, metal artifacts. Removing the implants before the pre-contrast scan would naturally remove the impact of scatter radiation in regards to angiogenic morphometry.

However, alterations of the vasculature through such a removal before the permanent setting with a silicone rubber contrast agent cannot be ruled out. Moreover, streaks and dark bands are likely to occur here as well, as both the mineralized bone tissue and the contrast agent possess high density, thus resulting in further artifacts [195].

Another limitation of µCT imaging is the blurring that occurs near the limits of spatial resolution due to nonlinear partial volume effects [196]. This can lead to either false connections between closely lying vessels or an apparent loss of vessels of less than a certain diameter, in this case, 15.6 µm.

Conclusion

In conclusion and within the limitations of this study, it could be shown that there was significant vessel sprouting in the early healing stage, depending on the force applied to the implants. This included elevated levels of V.Th. and VV/TV in higher loading groups. Furthermore, in 2W animals, VV/TV was shown to be increased in proximal sectors and V.Sp. in distal sectors, whereas in 8W animals, VV/TV was significantly higher in distal sectors, which might indicate that angiogenic activity in compression zones precedes that of tensile zones.

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