Three-dimensional microstructural properties of regenerated mineralizing tissue after PTH (1–34) treatment in a rabbit tibial lengthening model


1Orthopaedic Division of Northern Denmark, Aalborg University Hospital, University of Aarhus, Aalborg, Denmark; 2Institute of Anatomy, University of Aarhus, Aarhus, Denmark; 3Trauma Center Murnau, Murnau, Germany; 4Sector for Limb Reconstruction and Paediatric Orthopaedics, Aalborg University Hospital, Aalborg, Denmark; 5Department of Orthopaedics, Sector for Sports Medicine, Aarhus University Hospital, Aarhus, Denmark; 6Orthopaedic Research Laboratory, Department of Orthopaedics, Aarhus University Hospital, Department E, Aarhus, Denmark

Abstract

Since the approval of parathyroid hormone (PTH) as treatment for osteoporosis, PTH has increasingly been investigated for bone repair and regeneration. The aim of the study was to investigate the effects of intermittent PTH treatment on the microstructure of regenerated mineralizing tissue after distraction osteogenesis in rabbits. After tibial mid-diaphyseal osteotomy the callus was distracted 1 mm/day for 10 days. 72 rabbits were divided in to 3 groups, which daily received a PTH (1–34) 25 μg/kg injection for 30 days; a saline injection for 10 days and a PTH injection for 20 days; or a saline injection for 30 days. The microstructure of the regenerate was assessed by micro computed tomography (μCT). In all 51 obtained specimens were evaluated morphometrically using three different volumes of interests. The results showed that treatment with PTH during distraction osteogenesis resulted in a significantly higher trabecular number, a more isotropic trabecular orientation, a higher connectivity density, and a higher mineralizing tissue mass. We also found that distraction calluses treated with PTH were more mature than the non-treated. Conclusion: treatment with PTH resulted in an enhanced microstructure of the newly regenerated mineralizing tissue indicating that PTH has a potential role as a stimulating agent during distraction osteogenesis.

Keywords: Distraction Osteogenesis, Newly Regenerate Mineralizing Tissue, Parathyroid Hormone, Rabbits

Introduction

Parathyroid hormone (PTH) plays a large role in the bone metabolism, which it is able to affect in an either catabolic or anabolic way. Thus, intermittently administered human PTH is a strong bone anabolic treatment regimen1-2. It is widely recognized that treatment with PTH effectively increase bone mineral density (BMD) and prevent fractures in patients with osteoporosis3. In a large clinical trial of postmenopausal women with osteoporosis, treatment with PTH injections has demonstrated reduced risk of new vertebral and non-vertebral fractures by more than 50%4. Furthermore, PTH treatment increases BMD and improves both cortical and trabecular bone microarchitecture in humans5-8. Moreover, the improvements in trabecular and cortical bone structure induced by treatment with PTH appear to be one of the mechanisms for the fracture risk reduction9.

During the past few years there has been an increasing interest in the biology of bone repair and potential technologies for enhancing bone healing and regeneration. Several studies have demonstrated that treatment with PTH induced accelerated fracture healing in both rats10,15 and monkeys16. However, at present, knowledge on the effects of PTH treatment on newly regenerated bone after distraction osteogenesis is very limited. Seebach et al. reported enhanced mechanical strength and density of new bone after distraction osteogenesis in rats undergoing PTH treatment17. However, rat cortical bone is limited as a model of human cortical bone as rats has very little intracortical bone remodelling18,21, and as rats show a stronger...
bone anabolic response to PTH than humans²²,²³. Therefore, the effects of PTH on bone regeneration after distraction osteogenesis needs to be investigated in an animal model with intracortical bone remodelling. Furthermore, the microstructure of new regenerated bone after distraction osteogenesis has not previously been studied in PTH treated animals.

Distraction osteogenesis is a process for lengthening long bones where a slow, incremental distraction of the fracture callus is used to stimulate and prolong active new bone formation²⁴,²⁵. Gradual bone lengthening by distraction is increasingly being used routinely in orthopaedic surgery. Thus, callus distraction has been a standard procedure for leg lengthening, treatment of bone defects, malalignments, non-union fractures, and treatment of various structural deformities²⁶-²⁸. In humans, the process of new bone formation and consolidation, to a level where it has sufficient strength for weight bearing, requires months to years. Therefore, it is of interest to identify treatment regimens that is able to enhance bone formation in distraction osteogenesis so that the overall treatment duration can be reduced as much as possible.

During distraction osteogenesis woven bone is formed within the gap by intramembranous and endochondral ossifications²⁹. In woven bone the collagen fibrils are laid down in an a disorganized manner³⁰ and form new bone trabeculae, which during the distraction are orientated along the direction of distraction and later remodelled into an interconnected cancellous bone network²⁹.

The microstructure was investigated using micro computed tomography (µCT) rather than conventional histomorphometry as µCT makes it possible to investigate the microstructure and the mechanical properties of the same bone sample. In addition, it has been shown that µCT is an excellent replacement for conventional static histomorphometry³¹.

Recently, we have shown that treatment with PTH during distraction osteogenesis in rabbits resulted in significantly higher regenerate callus volume, bone mineral content, and bending strength³². Therefore, the aim of the present study was to investigate whether daily injections with human PTH would also improve the microarchitecture of the newly regenerated mineralizing tissue during distraction osteogenesis in rabbits.

Materials and methods

Animals

Seventy two 7-months-old (range 6-8 months) skeletally mature female New Zealand White rabbits with body weights of 3962±289 g were randomly divided into three groups. The rabbits were housed separately in standard cages in a temperature controlled room (21±2°C), with free access to food and water. The experiment was approved by the Danish Committee on Animal Experimentation.

PTH treatment regimens

The animals in the first group (PTH) were treated with a daily s.c. injection of 25 μg/kg b.w. PTH (human PTH (1-34), Bachem, Bubendorf, Switzerland) during both the lengthening and the consolidation period (30 days) (PTH group). The animals in the second group (vehicle + PTH) received a daily s.c. injection of vehicle during the lengthening period (10 days) and a daily s.c. injection of 25 μg/kg b.w PTH during the consolidation period (20 days) (saline+PTH group). The animals in the third group (control) received a daily s.c. injection with vehicle (30 days). The PTH dosage was established through a pilot study³³.

Surgical procedure

Surgery and distraction was performed using the protocol, introduced by Schumacher et al.³⁴ and later modified by Bundgaard³⁵. In brief: The right tibia was approached through a curved longitudinal skin incision. The fascia was cut, the muscles separated, and the anterior medial surface of the tibia was opened. The periosteum was incised longitudinally and carefully retracted. Four holes were drilled with a 2 mm drill and self-taping screws (Orthofix M300 or M301, Bussolenga, Italy) were inserted. The periosteum was protected and an osteotomy was performed at the mid-diaphysis just below the tibiofibular junction. A monolateral external fixator Orthofix M100 (Orthofix, Bussolenga, Italy) was mounted. The periosteum and muscular fascia were then closed over the bone with interrupted 4-0 sutures, and 4-0 sutures were used to close the skin. Postoperatively Buprenorphinum (Temgesic) was administered s.c. at a dose of 0.04 mg/kg twice a day in order to control post-operative and lengthening induced pain until 15 days after surgery, after which the dose was gradually reduced to 0 over the following 3 days. The surgical wound was inspected daily for signs of infection. Antibiotics were not used in the study. The animals were weight bearing and mobile at the day of the surgery.

Distraction protocol

The distraction was initiated 5 days postoperatively with a distraction rate of 1 mm/day (0.5 mm in two increments with at least 6 hours between the increments) and continued for 10 days. The distraction was followed by a 20 days consolidation period. Radiographs were taken under sedation 5, 14, and 28 days postoperatively in order to monitor bone formation and the fixation of the distraction device.

Specimen preparation

The animals were killed 5 weeks after the operation with an intravenous barbiturate overdose. The tibia of both legs were disarticulated, dissected free, carefully cleaned, radiographed in two planes, and then frozen at -20°C until the subsequent analyses.

An approximately 40-mm-long bone specimen containing the regenerated bone was obtained from the elongated tibia by sawing between the first and second screw hole and between the third and fourth screw hole with a diamond precision-parallel saw (Exakt Apparatebau GmbH, Norderstedt, Germany). The length axis of the obtained specimen was parallel to the tibia length axis.
Micro computed tomography

The bone specimens were scanned in a desktop μCT scanner (μ-CT 40; Scanco Medical AG, Brütisellen, Switzerland) at a resolution of 20x20x20 μm³. The acquired 3D data sets included the entire regenerate with approximately 2 mm of the old cortical bone at the proximal and distal end. Thus, each 3D data set consisted of approximately 600 transverse μCT slices of 1024x1024 pixels. After μCT scanning the resulting 3D data set was segmented with a fixed threshold filter using the software supplied with the scanner

For the subsequent analysis, smaller volumes of interests (VOI) were selected (Figure 1). Three VOIs with a height of 6 mm, but with different diameters were defined. All three VOIs were located in the regenerated callus so that they included only newly regenerated tissue and no old cortical bone. VOI₁ was defined by the outer boundary of the regenerated callus thereby including the entire central regenerate callus (Figures 1 and 2). VOI₂ was defined by the outer contour of the native tibial mid-diaphysis, thereby representing a region, which closely resembles the intact tibial shaft (Figure 3). VOI₃ was located in the new callus between the “cortex” and the “medullary canal” in order to selectively evaluate the microstructure of the new trabecular bone (Figure 4).

The microstructure of mineralized tissue was quantified with the software supplied with the μCT scanner. The bone volume fraction (BV/TV) was determined as the volume of bone tissue (BV) divided by the total volume of the VOI (TV). Trabecular number (Tb.N*), thickness (Tb.Th*), and separation (Tb.Sp*) were obtained directly from the trabecular network without model assumptions as described in detail by Hildebrand & Rüegsegger. In brief: Tb.Th* was determined as the diameter of spheres filling the bone structures, Tb.Sp* was determined as the diameter of spheres filling the marrow spaces, and Tb.N* was determined as the inverse of the mean distances of the skeletonized bone structure. The connectivity density (CD), which is an expression of the connectedness of the trabecular bone network, was determined using a method based on Euler numbers as described by Odgaard & Gundersen. The degree of anisotropy (DA), which quantify how oriented (or disoriented) the trabeculae are in the three spatial directions, was determined using the mean intercept length method as described by Rüegsegger et al.

Statistics

The Statistical analysis was performed using Stata (Stata Corporation, College Station, Texas, USA). Differences between the groups were tested by analysis of variance (ANOVA) with a t-test for post-hoc analysis. Before the analysis all data were tested for normal distribution and homogeneity of variances, and when these conditions were not fulfilled, the analysis was performed either on logarithmically transformed data or using the...
Figure 2. Sample μCT reconstruction images of VOI₁ depicting the microstructure of new trabecular bone obtained from a PTH-treated animal (A, B, and C) and a control animal (D, E, and F). VOI₁ was defined by the outer boundary of the regenerated callus, thereby including the entire central regenerate callus. A differentiation into medullar and cortical regions is clearly seen in the PTH-treated regenerate structure (A, B, C), whereas in the control group sample (D, E, F) the new trabecular bone is distributed over almost the whole callus without any clear zones.

Figure 3. Sample μCT reconstruction images of VOI₂ depicting the microstructure of new trabecular bone obtained from a PTH-treated animal (A) and a control animal (B). VOI₂ was defined by the outer contour of the native tibial mid-diaphysis, thereby representing a region close in size and location to the primary tibia shaft.
Kruskall-Wallis test followed by a Wilcoxon-Mann-Whitney test for post-hoc analysis. The results are presented as untransformed data as means±standard deviations (SD), and the used statistical method is indicated in the tables. A difference was considered significant at p<0.05.

Results

Twenty one rabbits were excluded from the study: eighteen with a tibial fracture through one of the fixation screw holes, two died due to repeated anaesthesia during x-ray examination, and one died for unknown reasons. In all 51 rabbits of 72 rabbits completed the experiment and were included in the analyses, with the following group distribution: 19 animals in the PTH group, 17 animals in the vehicle+PTH group, and 15 animals in the control group. During the experiment there were no signs of infection except a few mild wound-healing delays due to self-extraction of the operation suture requiring no treatment.

All rabbits lost weight during the study, on average 365±199 g. The animals in the PTH group lost 10% of their initial weight, the animals in the vehicle+PTH group lost 11% of their initial weight, and the animals in the control group lost 7% of their initial weight.

The length gained during the distraction was almost equal in all three groups. The average of the indirect measurement of the gained length (the length between the second and third screw holes) was 27.7±0.6 mm in the PTH group, 27.8±0.9 mm in the vehicle+PTH group, and 27.4±0.8 mm in the control group. In all the animals the tibia was lengthened approximately 10 mm (approximately 10% of the initial tibia length).

Micro computed tomography (Table 1-3)

3D data sets of VOI1 and VOI2 were obtained for 50 of the scanned 51 tibiae, that is: 18 in the PTH group, 17 in the vehicle+PTH group, and 15 in the control group. Due to technical problems with the μCT scanner only 46 images were obtained of VOI3 resulting in 16 samples in the PTH group, 17 in the vehicle+PTH group, and 13 in the control group. However, we considered that the remaining number of samples and the distribution of the samples between the groups were sufficient for including VOI3 in the analysis.

Representative three-dimensionally reconstructed images of all three VOIs are shown in Figures 2-4 illustrating the differences in microstructure between the groups.

A visual evaluation of the 3D reconstructed mineralizing tissue obtained with VOI1 suggests that animals treated with PTH had substantially larger and more mature calluses than control animals. By use of interactive 3D visualization it was established that the calluses of the PTH treated animals had a structure similar to that of diaphyseal bone, i.e. have a “medullary” space centrally, a very dense “cortical” bone-like structure peripherally, and a large region of newly formed trabecular bone in-between. In contrast, the calluses from the control group were substantially smaller and without clearly detectable regions in the microstructure. This indicates that the calluses of the PTH treated animals were more mature than the calluses of the control animals, where the new trabecular bone was remaining centrally. Moreover, the visual inspection indicated that the calluses of the PTH treated animals had many well connected trabeculae, whereas the calluses from the control group have fewer trabeculae with a more disoriented structure.
Table 1. Results of 3D microarchitecture parameters of new-trabecular bone in VOI1. VOI1 was defined by the outer boundary of the regenerated callus thereby including the entire central regenerate callus.

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<tr>
<th></th>
<th>Mean±SD</th>
<th>p-value</th>
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<tr>
<td></td>
<td>PTH</td>
<td>Vehicle+PTH</td>
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<td></td>
<td>(n=18)</td>
<td>(n=17)</td>
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<tr>
<td>BV (mm(^3))</td>
<td>258±79.6</td>
<td>260±104</td>
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<tr>
<td>TV (mm(^3))</td>
<td>776±165</td>
<td>726±238</td>
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<td>BV/TV</td>
<td>0.36±0.15</td>
<td>0.36±0.14</td>
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<td>CD (mm(^3))</td>
<td>61±19</td>
<td>75±24</td>
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<td>Tb.N*(mm(^{-1}))</td>
<td>3.5±0.95</td>
<td>3.2±1.4</td>
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<td>Tb.Th*(mm)</td>
<td>0.15±0.04</td>
<td>0.15±0.03</td>
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<tr>
<td>Tb.Sp*(mm)</td>
<td>0.36±0.16</td>
<td>0.47±0.26</td>
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<tr>
<td>DA</td>
<td>1.38±0.11</td>
<td>1.39±0.11</td>
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Table 2. Results of 3D microarchitecture parameters of new-trabecular bone in VOI2. VOI2 was defined by the outer contour of the native tibial mid-diaphysis, thereby representing a region, which closely resembles the intact tibial shaft.

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<td>PTH</td>
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<td>(n=17)</td>
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<tr>
<td>BV (mm(^3))</td>
<td>46±36</td>
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<td>TV (mm(^3))</td>
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<tr>
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<td>CD (mm(^3))</td>
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<td>Tb.N*(mm(^{-1}))</td>
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<tr>
<td>Tb.Th*(mm)</td>
<td>0.099±0.04</td>
<td>0.11±0.03</td>
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<tr>
<td>Tb.Sp*(mm)</td>
<td>0.39±0.18</td>
<td>0.52±0.41</td>
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<tr>
<td>DA</td>
<td>1.5±0.13</td>
<td>1.6±0.12</td>
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Table 3. Results of 3D microarchitecture parameters of new-trabecular bone in VOI3. VOI3 was located in the new callus between the “cortex” and the “medullary canal” in order to selectively evaluate the microstructure of the new-trabecular bone.

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<th></th>
<th>Mean±SD</th>
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<tr>
<td></td>
<td>PTH</td>
<td>Saline+PTH</td>
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<td></td>
<td>(n=16)</td>
<td>(n=17)</td>
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<tr>
<td>BV (mm(^3))</td>
<td>18±6.4</td>
<td>19±6.3</td>
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<td>TV (mm(^3))</td>
<td>35±1.5</td>
<td>35±0</td>
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<tr>
<td>BV/TV</td>
<td>0.52±0.17</td>
<td>0.54±0.17</td>
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<tr>
<td>CD (mm(^3))</td>
<td>96±30</td>
<td>105±37</td>
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<tr>
<td>Tb.N*(mm(^{-1}))</td>
<td>5.8±0.57</td>
<td>5.9±0.89</td>
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<tr>
<td>Tb.Th*(mm)</td>
<td>0.13±0.04</td>
<td>0.14±0.05</td>
</tr>
<tr>
<td>Tb.Sp*(mm)</td>
<td>0.13±0.04</td>
<td>0.14±0.06</td>
</tr>
<tr>
<td>DA</td>
<td>1.76±0.15</td>
<td>1.67±0.16</td>
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(Th, Th*) and trabecular separation (Th, Sp*) was significantly lower in the two PTH treated groups than in the control group. This indicates that substantially more, but slightly thinner trabeculae were formed in the PTH-treated animals than in the control animals. The vehicle+PTH group had a significantly higher connectivity density than the control group. In addition, the PTH-treated animals had a significantly more isotropic trabecular network than the control animals. No significant differences were found between the two PTH-treated groups in any of the determined structural parameters.

VOI1 (Table 2). The microstructural parameters of VOI1 indicated that all PTH-treated animals had more but thinner trabeculae than the control animals. In addition, the trabeculae were more isotropic oriented in the PTH-treated animals than in control animals, but the differences in bone volume density and bone volume, were not statistical significant between the groups.

VOI2 (Table 3). The microstructural parameters of VOI2 showed that the PTH-treated animals had a significantly higher Tb.N* than the control animals, indicating that more trabeculae had been formed in the regenerate bone in PTH-treated animals. The trabeculae were also thinner in PTH-treated animals, but - in contrast to VOI1 and VOI3 - this difference did not reach the level of significance. The trabecular network in the regenerated callus was more isotropic in PTH-treated animals than in control animals. However, this difference was only significant for the vehicle+PTH group. Additionally, the connectivity density was significantly higher in both PTH treated groups and the trabeculae were significantly more isotropic in the vehicle+PTH group than in the control group. As in the two other VOIs, the bone volume fraction did not differ significantly between the groups in VOI3.

Discussion

The purpose of the study was to investigate whether intermittent PTH treatment affects the microstructure of newly regenerated mineralizing tissue after distraction osteogenesis in animals with intracortical remodelling. In order to fulfil this aim, specimens of distracted and regenerated rabbit tibiae were analyzed using μCT. It was shown that the newly formed mineralizing tissue after intermittent PTH treatment had significantly more trabeculae that were better connected and more isotropic arranged, and the bone volume of the regenerated callus was higher compared to control animals, which confirmed our hypothesis. In addition, we found preliminary evidence, which suggests that the newly regenerated callus was more mature (i.e. is remodelled further in the direction of cortical bone) in animals treated with PTH than in control animals.

To the best of our knowledge previously only one other experimental study has been conducted investigating the effects of PTH on distraction osteogenesis\(^\text{27}\). However, that study was conducted using rats, which have limited or no intracortical bone remodelling. The rabbit is one of the smallest animals in which intracortical remodelling similar to humans occurs\(^\text{40}\). Consequently, the rabbit was selected as a suitable model to examine the effects of PTH-treatment on the microarchitecture of the new regenerated mineralizing tissue.

Previously, we have shown that treatment with PTH during distraction osteogenesis in rabbits resulted in significantly stronger regenerated callus compared with untreated control animals\(^\text{32}\). In that study we showed, using a three-point bending test, that the rabbits treated with PTH during the lengthening and consolidation period had a bending strength of 334 N, the rabbits treated with PTH during the consolidation period only had a bending strength of 338 N, whereas the rabbits treated with vehicle had a bending strength of 260 N. The animals in both PTH treated groups had significantly (p<0.001) stronger regenerated bone than the untreated control animals. In addition, we showed that the strength of the regenerated mineralized tissue did not differ significantly between the animals treated with PTH during both the lengthening and the consolidating period and the animals treated with PTH during the consolidating period only.

In the present study we have investigated whether the PTH treatment also lead to an improved microstructure in the distraction callus. We analysed the microstructure of the distraction callus using three different VOIs. The analysis of the three VOI’s showed that the PTH treatment resulted in regenerated bone with higher trabecular number and connectivity density, and lower trabecular separation and degree of anisotropy.

Richards et al. studied regenerated bone in lengthened rabbit tibiae as a function of time post surgery using histomorphometry\(^\text{41}\). The findings of Richard et al. are in general agreement with the results of the present study.

In the present study the bone volume of the regenerated mineralizing tissue was significantly higher in PTH-treated animals, whereas the bone volume fraction was not influenced by the PTH treatment. The effect of PTH treatment on bone volume fraction reported in the literature is somewhat inconsistent and merits a brief discussion. In rats undergoing distraction osteogenesis PTH treatment resulted in a 35% higher regenerated bone volume fraction\(^\text{35}\). In monkeys vertebral cancellous bone volume was significantly higher in PTH treated ovariectomized animals than in untreated ovariectomized animals\(^\text{45}\). However, in intact rabbits the bone volume fraction of the lumbar vertebra did not change after PTH treatment despite evidence for an increased bone turnover\(^\text{39}\). Moreover, changes in bone volume fraction of the iliac crest and lumbar vertebrae after PTH treatment were not detectable in beagle dogs\(^\text{44,44}\).

In the newly generated trabecular bone (VOI3) we found that the PTH treatment resulted in a significantly higher trabecular number and connectivity density indicating a better connected trabecular network. In addition, we found that the PTH treatment did not influence the trabecular thickness, which is in agreement with the findings of the tibial lengthening study by Seebach et al. conducted in rats\(^\text{17}\).

In the present study we have chosen to use a uniform threshold for all samples, which is an advantageous procedure for obtaining comparable results\(^\text{46,45}\), even though other researchers have argued differently\(^\text{46-48}\). However, Müller et al. concluded that the use of a uniform threshold is an adequate procedure for evaluation of trabecular bone\(^\text{45}\). Moreover, in the present...
study we found that applying individual thresholds for each sample would have been very difficult, as we observed density variations not only between the specimens but also within a specimen.

We have chosen two different periods of PTH administration in order to investigate whether the effect of PTH treatment during lengthening and consolidation differ from the effect of PTH treatment during the consolidation period only. In the present study none of the analyzed bone parameters determined from VOI differed significantly between the two PTH treatment regimens. The rabbit tibial distraction study by Richards et al. demonstrated that the bone formation in the gap was most active between 18 and 24 days after the operation, and they suggest that “interventions designed to enhance the process should occur during this time window.” Consequently, their results in combination with the results of the present study suggest that the PTH induced stimulation of bone formation should be initiated during the later stages of the lengthening or at the beginning of the consolidation period, as the early start with PTH treatment did not markedly influenced the new bone formation.

In conclusion, the present study demonstrates for the first time that systemic PTH treatment resulted in an improvement of the microarchitecture of regenerated mineralizing tissue in distraction osteogenesis. The data showed that treatment with PTH resulted in a mineralizing tissue with a significantly higher trabecular number, a more isotropic trabecular orientation, a higher connectivity density, and an enhanced regenerated callus volume. Moreover, we found preliminary evidence, which suggests that intermittent PTH treatment accelerated callus remodelling into a more mature callus. Consequently, although the scientific knowledge is at present limited due to the very few experimental studies performed in this field, the present study nevertheless indicates that PTH treatment has a potential to enhance bone remodelling during distraction osteogenesis.

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