CHAPTER 5

EFFECTS OF COMBINED PEAK POWER AND ENDURANCE TRAINING ON THE OSTEOGENIC RESPONSE IN RAT Tibia

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ABSTRACT

Purpose: Mechanical loading of the musculo-skeletal system plays an important role in the adaptation of muscle performance and bone mass. In contrast to the interference effect between peak power and endurance training on the adaptive response of muscle tissue, in bone tissue, such interaction between training modalities may not exist and combined peak power training and endurance training may even have a cumulative effect on bone mass. We hypothesised that combined peak power and endurance training enhances the osteogenic response as compared to one type of training alone, provided that the training sessions are separated by a rest period.

Methods: 38 rats were subjected to either 6 weeks of peak power (PT, n=10), endurance (ET, n=10), combined peak power and endurance training (PET, n=10) or no-training (control, n=8). mRNA expression levels of insulin-like growth factor 1Ea (IGF-1Ea), osteoprotegerin (OPG), receptor activator of nuclear factor-κB ligand (RANKL), tumour necrosis factor α (TNF-α), interleukin 1α (IL-1α), IL-1β, IL-6, alkaline phosphatase (ALP), osteocalcin and collagen 1α2, were analysed. In addition, we analysed trabecular bone turnover by histomorphometry.

Results: IGF-1Ea and RANKL mRNA levels were significantly lower after PT than after ET. Gene expression levels of OPG were significantly lower after PT compared to those of controls, ET and PET. OPG/RANKL ratio remained unchanged after training. mRNA expression levels of cytokines, ALP, osteocalcin and collagen 1α2, as well as bone turnover measured by histomorphometry were unchanged after training. After PET, the variation within the gene expression levels (except for expression levels of cytokines) were much larger than that within the other groups.

Conclusions: The large variation in the gene expression observed after PET suggests that approximately in half of the rats the transcriptional response of genes involved in bone turnover was prolonged. The lower expression levels of osteo-anabolic factors after peak power training were neutralised when additional endurance training was performed. This suggests that in the long-term combined peak power and endurance training may be a useful strategy to enhance the osteogenic response.
INTRODUCTION
Mechanical loading of the musculo-skeletal system plays an important role in the adaptation of muscle performance and bone mass. An inactive lifestyle, common in older people reduces mechanical loading on muscles causing muscle weakness. Not only maximal force is reduced in older people, but also the oxidative capacity is diminished as a result of reduced mitochondrial function [66,81]. Accordingly, the ability to move is impaired which implicates an even more sedentary lifestyle. As a consequence, mechanical loading of bones is reduced, resulting in a lower rate of bone formation and reduced bone mineral density (BMD).

To maintain a healthy lifestyle, the muscle force generating capacity as well as the oxidative capacity need to be improved and hence subjects should train muscles for both traits. However, within a muscle fibre, evidence suggests that the adaptive responses to combined peak power and endurance training interfere with one another and instead of simultaneously increasing maximal muscle force and oxidative capacity, adaptation of both traits is attenuated [4,26,43,44,191]. Note that although combined peak power and endurance training may not increase maximal muscle force to the same extent as after peak power training alone, combined training was still shown to be effective to increase both maximal force and oxidative capacity [65,155]. In contrast to the interference effect between peak power and endurance training on the adaptive response within skeletal muscle, in bone, such an interaction between training modalities may not exist, because the metabolic demand during exercise is lower in bone cells than that in muscle cells. As such, combined peak power training and endurance training may even have a cumulative effect on bone mass.

Very little is known about combined effects of peak power training and endurance training on bone remodelling. In general, high forces exerted onto the bone, which are attained with peak power training, are very effective to increase bone mass [17,158]. However, in young growing rats, low-impact training, such as low velocity treadmill running, was also shown to increase bone mass by reducing bone resorption [54,55] or stimulate bone formation [73,78,82,83]. Whether this type of training does also enhance the osteogenic response in adult rats is still under debate and may be site-specific [9,34,193]. In middle-aged rats, treadmill running was suggested to attenuate the age-related bone loss by reducing bone resorption [30]. Besides mechanical loading onto bone during training, bone turnover may also be altered via
paracrine and/or endocrine pathways. In skeletal muscles, endurance exercise was shown to up-regulate the expression levels of interleukin 6 (IL-6) and IL-10, as well as to down-regulate the expression levels of pro-inflammatory cytokines such as tumour necrosis factor α and IL-1, inducing an anti-inflammatory response [107,135]. Possibly, additional endurance training on top of peak power training may create an osteogenic environment and may thereby affect bone turnover via paracrine and/or endocrine signalling. Since in osteoporotic older people, high-impact loading is dissuaded, as this increases the risk of (vertebral) fractures [129], training with mainly low-impact exercises may be more appropriate to increase bone formation in these people. This raises the question whether additional endurance training on top of peak power training enhances the osteogenic response and may be a useful training strategy to increase bone formation with a minimal amount of high-impact loading.

Bone cells have shown to become desensitised rapidly to mechanical loading [17,186] and 40 daily jumps in rats did not increase bone mass more than as few as 5-10 jumps per day [186]. Rest periods between the loading sessions are required to restore mechano-sensitivity of bone cells, resulting in an enhanced osteogenic response compared to loading without rest periods [148,149,150]. Therefore, when developing training regimes aiming to increase the osteogenic response, not only the magnitude of the loading should be taken into consideration but also the rest periods between the loading sessions. A recovery period of 8 hours was reported to be sufficient to restore mechano-sensitivity of bone cells to loading [149]. This indicates that a low-impact training following a high-impact training with a sufficiently long time interval may be effective to increase bone formation. To the best of our knowledge, effects of a combination of peak power training and endurance training on bone have not been studied yet.

The aim of the present study was to investigate the effects of combined peak power and endurance training on the osteogenic response in the rat tibia, while separating the training sessions by an 8 hour rest period in order to maintain or enhance bone sensitivity. To obtain insight into the effects of different types of training on regulatory factors of bone turnover, mRNA expression levels of insulin-like growth factor 1Ea (IGF-1Ea), osteoprotegerin (OPG) and receptor activator of nuclear factor-κB ligand (RANKL) and their ratio were analysed. In addition, mRNA expression levels of cytokines and markers for bone formation were analysed as well as measures of
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trabecular bone turnover using histomorphometry. We hypothesised that peak power training stimulates bone formation by increasing the mRNA expression levels of osteo-anabolic factors and bone formation rate. For endurance training, we expected an attenuation in bone resorption by reducing mRNA expression levels of osteo-catabolic factors as well as a reduction in the number of osteoclasts. We questioned whether endurance exercise also increases bone formation in adult rats as shown in young growing rats. Finally, we hypothesised that combined peak power and endurance training is more effective to increased the osteogenic response compared to either one type of training only.

Materials and methods

Animal care and experimental design

The experiment was approved by the Animal Experiments Committee of the VU University Amsterdam and animals were kept according to the guidelines of animal care. The study was conducted with 38 female Wistar rats at the age of 10 weeks with food and water provided ad libitum. The rats were subjected to either peak power training (PT, n=10), endurance training (ET, n=10), combined peak power and endurance training (PET, n=10) or no-training (control, n=8). In order to train the rats during their active period of the day, the 12-h light:12-h dark cycle was reversed. During a three week acclimatisation period, rats of the training groups were familiarised with running and/or sprinting on a motor driven treadmill. Following this, the 6 week training period started, during which the rats of the PT and ET group performed 5 training sessions per week (one session per day) and the PET group performed 10 training sessions per week (two sessions per day). For studying effects on bone mineralisation, 25 mg/kg of tetracycline was injected intraperitoneally 9 and 2 days before sacrificing. To standardise the time between the last training session (peak power training for the PT group and endurance training for the ET and PET group) and sampling of the tibia to exclude acute training effects, the last training was performed 22 hours (range 20.0-23.8 hours) before sampling of the bone. The right tibia was used for histomorphometry, whereas the left tibia was used to study gene expression.

Training protocols

Peak power training consisted of 10 sprints of 15 seconds at maximal attainable ve-
locity (in gallop) with 3 minutes of rest between the sprints. Compressive strain of the tibia was shown to be higher when the animals were in gallop [10]. In order to increase the forces on the hind limbs, the slope of the treadmill was increased progressively up to 40%. Pilot data with force plate measurements showed that forces on the hind limbs increase with increasing slopes (unpublished data from our lab). Endurance training consisted of treadmill running, in which the duration was progressively increased up to 45 minutes, as well as the inclination and velocity were progressively increased up to 10% and 26 m/min (trotting), respectively. The PET group performed both training sessions on the same day (PT in the morning and ET 8 hours later).

Histomorphometry

Tissue preparation – After cleaning the right tibia from the soft tissue, the non-decalcified tibia was fixed in cold 4% phosphate-buffered formaldehyde, dehydrated in graded ethanol, and embedded in 80% methylmethacrylate (BDH Chemicals, Poole, England) supplemented with 20% dibuthylphthalate (Merck, Darmstadt, Germany), 8 g/L lucidol CH-50L (Akzo Nobel, Deventer, The Netherlands) and 22 ml/10 ml N,N dimethyl-p-toluidine (Merck). Longitudinal sections were cut (5 μm thick) using a Polycut 2500 S microtome (Reichert-Jung, Nussloch, Germany). Tetracyline labels were measured in unstained sections using fluorescence microscopy. To visualise osteoclasts, sections were stained for tartrate-resistant acid phosphatase (TRAP) [13]. The staining was performed on sections which were mounted on glass slides.

Analyses – Histomorphometry was performed in the secondary spongiosa of the trabecular bone using NIS-Elements AR 2.10 (Nikon GmbH). Images of the TRAP staining were captured at x200 magnification and images of the unstained sections (for the tetracycline labels) at x100 magnification. The measures were performed according to the American Society of Bone and Mineral Research (ASBMR) nomenclature [35]. Trabecular bone volume (bone volume/total volume - BV/TV) is defined as a percentage trabecular bone area of the total area of the secondary spongiosa. As a measure for bone resorption, the surface of trabecular bone which was covered by TRAP-positive osteoclasts was measured and expressed as percentage of the total trabecular surface. As an index for bone formation, single and double tetracycline labelled (mineralising) trabecular bone surface as well as interlabel width were measured under ultraviolet light. Following this, mineral apposition rate (MAR in μm/day)
and bone formation rate (BFR = ((double label + ½*single label) * MAR) in μm³/μm²/day) were calculated. All measurements were performed in duplicate by a single blinded investigator.

Quantitative polymerase chain reaction (qPCR)

Tissue preparation and RNA extraction – The tibia was cleaned from soft tissue and stored at -80°C until further analysis. One week before RNA was isolated, the tibia was stored in RNAlater® - ICE (Ambion, Life Technologies, Bleiswijk, The Netherlands) at -80°C. RNA was isolated from the shaft of the tibia. After cutting off the proximal and distal end of the tibia containing trabecular bone, the diaphysis was flushed with ice cold RNase free water to remove the bone marrow. The bone was grounded in liquid nitrogen using a freezer-mill (SPEX 6750, Glen Creston, Stanmore, England). Following this, the bone powder was incubated in Trizol (Invitrogen, Life Technologies, Bleiswijk, The Netherlands) for 1 hour at 37°C. After the first Trizol extraction, a chloroform/isomyl alcohol extraction was performed and followed by a second Trizol extraction according to the manufacturer’s instructions. Possible DNA contamination was removed by DNAse incubation (Promega, Leiden, The Netherlands). The pellet was dissolved in RNase free H₂O and stored at -80°C until further analysis.

Reverse transcription – Total RNA concentration and quality was measured using a bio-analyser (2100 Expert, Agilent Technologies, Inc.). Following this, 100 ng RNA was reversed transcribed using 10 ng/μl random primers (Roche, Almere, The Netherlands), 5 U/μl M-MLV Reverse Transcriptase (RT) and a mixture of 5 mM MgCl₂, 1x RT-buffer, 1 mM dNTPs each, 1 M betaine and 0.40 U/μl RNAsin (Promega). These samples (total volume of 20 μl) were incubated for 10 min at 25°C, 1 h at 37°C and 5 min at 95°C.

qPCR – In a total volume of 25 μl containing 3 μl cDNA, 300 nM primer and 12.5μl SYBR® Green Supermix (BioRad, Veenendaal, The Netherlands), qPCR reaction was performed in the iCycler system (BioRad), consisting of 40 amplification cycles (15 s at 95°C and 1 min at 60°C). mRNA levels were assessed for insulin-like growth factor 1 Ea (IGF-1Ea), osteoprotegerin (OPG), receptor activator of nuclear factor-κB ligand (RANKL), tumour necrosis factor α (TNF-α), interleukin 1α (IL-1α), IL-1β and IL-6, alkaline phosphatase, osteocalcin, collagen type 1α2. Hypoxanthinephophoribosyltransferase (HPRT) served as housekeeping genes. Primer sequences are shown in
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table 5.1. The threshold, set in the linear part of the curve, was identical for all genes. Expression levels were expressed relative to the housekeeping gene using the $2^{-\Delta Ct}$ method. All samples were analysed in triplicate.

Table 5.1. Sequences of the specific primers used in the quantitative PCR analyses.

<table>
<thead>
<tr>
<th>PCR primer sequence 5' --&gt; 3'</th>
<th>Target mRNA</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPRT GTGTCATCAGCGAAAGTGGA</td>
<td>TACTGGCCACATCAACAGGA</td>
<td></td>
<td></td>
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<tr>
<td>IGF-1Ea AAGCCTACAAAAGTCAGTCG</td>
<td>TCAAGTGTACTTCTTCTGAGTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPG ACGGTTTGCAAAAGATGTCC</td>
<td>GTGAGCTGCAGTTGGTGTGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RANKL ACCAGCATCAAATCCCAAG</td>
<td>GGACGCTAATTTTCCTACCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α GAGGTCAACCTGCCCAAGTA</td>
<td>GCTGGGTAGAGAACGGATGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1α AACCCAGATCAGCACCTCAC</td>
<td>TGATGAACTCCTGCTTGAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β CAGGAAGGCAGTGTCACTCA</td>
<td>AAAGAAGGTCTTTGGGTCCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6 CCGGAGAGGAGACTTCACAG</td>
<td>CAGAATTTGCAATCGCAACAC</td>
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<td></td>
</tr>
<tr>
<td>ALP GACAAGAAGCCCTACAGC</td>
<td>ACTGGGCCTGGTCAGTTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteocalcin TCTGCTCXTCTGCTGGCC</td>
<td>TCAGAGTCGCTGGGCTTTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagen 1α2 GCTGGGCTGATGGTGAC</td>
<td>TGGGGCCAGAAGGACCAG</td>
<td></td>
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</tr>
</tbody>
</table>

HPRT: hypoxanthinephophoribosyltransferase; IGF-1Ea: insulin-like growth factor 1Ea; OPG: osteoprotegerin; RANKL: receptor activator of nuclear factor-κB ligand; TNF-α: tumour necrosis factor α; IL: interleukin; ALP: alkaline phosphatase

Statistical analyses

SPSS version 18.0 (SPSS Inc., Chicago, USA) was used for data analyses. Kruskal-Wallis Test was performed to test for differences between the four groups. Mann-Whitney U Test was performed to compare the different groups when a significant main effect was observed. The p-value was adjusted using the Bonferroni correction method. The results are presented in mean±SE. Significance was reported as $p<0.05$.

RESULTS

In total, thirty rats were trained. Three rats (two of the PT group and one of the ET
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Effects of training on mRNA expression of bone turnover related factors

Regulatory factors of bone turnover – Peak power training was expected to increase the expression levels of IGF-1Ea and OPG. However, in contrast to our expectations, IGF-1Ea mRNA levels were significantly lower after PT compared to those in the ET group (Fig. 5.1A). Similarly, gene expression levels of OPG were significantly lower after PT compared to those of controls, ET and PET (Fig. 5.1B). This suggests that the peak power training performed in our study did not increase the osteogenic response but may have had an attenuating effect on bone formation. After ET and PET, expression levels of IGF-1Ea and OPG were not different from those of controls, suggesting that these two types of training did neither increase nor attenuate the osteogenic response compared to controls. The osteo-catabolic factor RANKL which stimulates osteoclastogenesis and bone resorption was expected to decrease in response to endurance training. In contrast to our expectation, after ET, RANKL mRNA levels were not different from those of controls (Fig. 5.1C), suggesting that this type of training did not enhance nor attenuate osteoclastogenesis. However, after PT, RANKL mRNA expression levels were significantly lower than after ET. This suggests that PT attenuated rather than stimulated osteoclastogenesis. After PET, RANKL mRNA levels did not differ from those of controls. In addition, OPG/RANKL ratio did not change after training (Fig. 5.1D). These data suggest that it is unlikely that the lower expression levels of OPG and RANKL after PT would have altered bone resorption 22 hours post-exercise.

Regarding the effects of PET, it should be noted that the variation of the expression levels in this training group was substantially larger than that of the other
groups. Approximately half of the rats of the PET groups showed values which were 1.5-2 times higher than the mean values of the other groups. This suggests that in half of the rats, the combination of peak power and endurance training may have a cumulative effect on the osteogenic response and may be effective to stimulate bone formation.

Figure 5.1. Effects of training on mRNA expression levels of regulatory genes involved in bone turnover in rat tibia. Effects of 6 weeks of peak power training (PT, n=8), endurance training (ET, n=7), combined peak power and endurance training (PET, n=10) or no-training (control, n=7) are shown on regulatory factors for osteogenesis: A) insulin-like growth factor 1Ea (IGF-1Ea), B) osteoprotegerin (OPG), C) receptor activator of nuclear factor-κB ligand (RANKL), and D) ratio OPG/RANKL. mRNA expression is relative to the housekeeping gene HPRT. All values are mean±SE. * = significant difference between groups (p<0.05)

Cytokines – Expression levels of inflammatory factors TNF-α, IL-1α/β, IL-6, which are known for their stimulatory effects on osteoclastogenesis, were expected to decrease in response to the endurance training. However, mRNA levels of TNF-α, IL-
1α/β, IL-6 did not differ between the four groups (Figs. 5.2A-D). These data suggest that the bone turnover rate was unlikely to be altered by osteo-catabolic cytokines after any type of training.

Figure 5.2. Effects of training on the mRNA expression levels of cytokines in rat tibia. Effects of 6 weeks of peak power training (PT, n=8), endurance training (ET, n=7), combined peak power and endurance training (PET, n=10) or no-training (control, n=7) are shown on cytokines: A) tumour necrosis factor α (TNF-α), B) interleukin 1α (IL-1α), C) IL-1β and D) IL-6. mRNA expression is relative to the housekeeping gene HPRT. All values are mean±SE.

Genes involved in the synthesis of bone matrix – Peak power training was expected to increase mRNA gene expression levels typically involved bone formation. In contrast to our expectations, we could not show any differences in mRNA expression levels of
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ALP, osteocalcin and collagen type 1 after different training modalities (Figs. 5.3A-C). This suggests, that although PT altered mRNA expression levels of osteo-anabolic and osteo-catabolic factors, these changes were unlikely to have affected the rate of bone formation.

![Graphs](image)

**Figure 5.3. Effects of training on the expression of genes involved in bone formation in rat tibia.** Effects of 6 weeks of peak power training (PT, n=8), endurance training (ET, n=7), combined peak power and endurance training (PET, n=10) or no-training (control, n=7) are shown on markers for bone formation: A) alkaline phosphatase, B) osteocalcin and C) collagen type 1α2. mRNA expression is relative to the housekeeping gene HPRT. All values are mean±SE.

**Effects of training on the bone volume and bone turnover**

After peak power training, we expected an increase in bone formation rate and mineralising surface, whereas endurance training was expected to decrease bone resorption as measured by the surface of trabecular bone covered by osteoclasts. These
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Changes could have eventually resulted in a larger total trabecular bone volume. Combined peak power and endurance training was expected to increase bone formation as well as decrease bone resorption, resulting in a higher bone volume compared to one type of training only. However, as shown in figure 5.4, total trabecular bone volume, osteoclast surface, mineral apposition rate, mineralising surface (as measured by tetracycline double labels) and bone formation rate were not different between groups. Thus, after 6 weeks of training, osteogenic response in trabecular bone was unlikely to be increased by the different training modalities.

Figure 5.4. Effects of training on structural changes in bone volume and bone turnover in rat tibia. Effects of 6 weeks of peak power training (PT, n=8), endurance training (ET, n=7), combined peak power and endurance training (PET, n=10) or no-training (control, n=7) are shown on A) trabecular bone volume (percentage trabecular bone volume/total volume, BV/TV), B) osteoclast surface (OcS) per bone surface (BS), C) mineral apposition rate (MAR), D) double labels (DL/BS) and E) bone formation rate (BFR). Rats were trained for 6 weeks 5 days per week performing either peak PT, ET or PET. All values are mean±SE.
DISCUSSION

The aim of this study was to test whether peak power training would stimulate bone formation and endurance training would attenuate bone resorption. In addition, we hypothesised that combined peak power and endurance training would enhance the osteogenic response compared to one type of training only. Here we show that, in contrast to our hypothesis, PT reduced mRNA expression levels of osteo-anabolic factors compared to those of ET. This suggests that PT had a negative effect on bone formation. Although ET did not have a negative effect on bone formation as was shown after PT, ET did not reduce bone resorption. For all analysed genes, the combination of peak power and endurance training, showed no differences with ET. This suggests that additional endurance training had counteracted the negative effects of PT. In addition, after PET, a high variation in mRNA expression levels of genes involved in bone formation were observed, suggesting that in half of the rats of the PET group, daily combined training for 6 weeks had a cumulative effect and may enhance the osteogenic response in the long-term.

Study limitations

A limitation of this study is that mRNA levels were only determined at one time point after the training. Since we were interested in changes in steady state mRNA levels rather than acute training effects, we sampled bone tissue 22 hours after the last training session. Generally, after mechanical loading of osteocytes-like cells in vitro, mRNA levels increase up to approximately 6 hours and return back to baseline levels after 24 hours [84]. However, if training is performed for a longer period of time, as performed in this study, steady state mRNA levels may increase and consequently also mRNA levels 22 hours after the last training session may be elevated. Such increases in steady state mRNA levels were shown within medial gastrocnemius muscle (GM) after the training modalities as imposed in this study [43,44]. If temporal training-induced changes in steady state mRNA levels in muscle and bone were similar, we would also expect to measure effects on steady state mRNA levels in bone. However, our measurements do not reveal earlier changes and transient effects in expression levels of osteo-anabolic and catabolic factors that may occur in response to training.

Another limitation is that the two different types of analyses (i.e. qPCR and histomorphometry) were performed at two different locations within the tibia. mRNA was
isolated from cortical bone, as RNA isolation from trabecular bone will always result in mRNA from a mixed population of cells including not only the required osteoblasts and osteocytes but also cells from the haematopoietic cell line as they are situated in the surrounding bone marrow. Since loading was applied to the whole tibia, we presume that both trabecular and cortical bone responded with similar changes in mRNA expression levels. However, differences in adaptive responses between cortical and trabecular bone cannot be excluded. Assuming that the adaptive response of bone to mechanical loading is similar for cortical as well as trabecular bone, changes in mRNA expression levels of bone remodelling factors will eventually result in altered histomorphometric indices for bone turnover. These data were assessed in trabecular bone. Since bone turnover rate in cortical bone is slower than in trabecular bone, effects of 6 weeks of training on bone volume, osteoclast surface and bone formation rate were expected in trabecular bone rather than in cortical bone.

**Effect of peak power training on the osteogenic response**

We hypothesised that after 6 weeks of PT, mRNA expression of osteo-anabolic factors would have been increased. However, lower IGF-1Ea mRNA levels were observed after 6 weeks of PT compared to those of ET. IGF-1 is considered an osteo-anabolic factor as it stimulates proliferation of pre-osteoblast and their differentiation into osteoblasts and as such increase the rate of synthesis of bone matrix [29,45]. In humans, serum IGF-1 levels correlate positively with BMD [90]. However, whether IGF-1 expression is increased by mechanical loading in vivo remains subject to controversy [14,143]. Four point bending of the rat tibia induced an increase in the expression of IGF-1 mRNA in osteocytes [143], whereas 6 weeks of treadmill running decreased IGF-1 protein in rat humerus [14]. This suggests that IGF-1 expression may depend on the type of mechanical loading and/or the duration of the study. The lower IGF-1 mRNA expression levels after PT were accompanied by reduced OPG mRNA expression levels after PT compared to those of ET, PET and controls, confirming the negative effect of PT on bone formation. However, also RANKL mRNA expression levels were lower after PT compared to those of ET, resulting in unaltered OPG/RANKL ratios. In contrast to our findings, several *in vitro* studies using osteoblastic cells as well as murine stromal cells reported increased OPG/RANKL mRNA ratios immediately after mechanical loading of these cells (as applied by pulsating fluid flow, vibration or stretching). This
was either due to a decrease in RANKL mRNA expression and/or an increase in OPG [92,93,96,97]. Our data suggest that although mRNA levels of osteo-anabolic factors were lower after PT, the osteoclast function was not enhanced by PT because of the unchanged OPG/RANKL ratio. In our study, expression levels of genes involved in the synthesis of bone matrix were unchanged. Therefore, in contrast to our hypothesis, 6 weeks PT did certainly not increase bone formation and if PT had any effect on the rate of bone turnover, it would likely have had an osteo-catabolic effect.

Effects of endurance training on the osteogenic response

In contrast to the expected attenuation of bone resorption, 6 weeks of ET did not reduce mRNA expression levels of osteo-catabolic factors. In addition, trabecular bone surface covered by osteoclasts was unaffected by ET. Besides the question whether ET would induce a decrease in bone resorption, we questioned whether ET may be effective to increase bone formation in adult rats, as in young growing rats, low intensity running exercise, similar to our ET, increased trabecular bone volume after 8 and 10 weeks of training [78,82]. However, these studies were performed with very young growing rats at the age of 4 weeks at the beginning of the training [78,82], whereas the rats in our study were 13 weeks at the beginning of the training. In contrast to young growing rats, ET in young adult rats did not increase bone formation. At a young age, before maturity, rats may show different adaptive responses to training compared to adult rats. In another study with adult rats (17 weeks), 12 weeks of endurance running exercise did not induce changes in the trabecular bone [193], which is in accordance to the findings of our study. A possible explanation for how mechanical loading may induce distinct adaptive responses in immature trabecular bone compared to mature trabecular bone is the force distribution within the primary and secondary spongiosa in combination with the longitudinal growth of the tibia [42]. In response to mechanical loading, forces exerted onto trabecular bone are highest within the primary spongiosa and are eventually transferred by trabeculae of the primary and secondary spongiosa onto the cortex of the tibia [42,185]. The mechanical loads become less with increasing distance from the growth plate (deloading of the secondary spongiosa) [42,185]. The number and thickness of trabeculae within the primary spongiosa may increase more in response to mechanical loading than those within the secondary spongiosa. By longitudinal growth of the tibia, primary
spongiosa shifts away from the growth plate and becomes secondary spongiosa [42]. The analyses of bone formation by histomorphometry in the studies mentioned previously [78,82,193], were all performed within the secondary spongiosa. In very young growing animals, the analysed trabeculae were likely to have sensed higher forces during the training period as they originate from the primary spongiosa in which the magnitude of the mechanical loading applied to trabeculae is higher than that in the secondary spongiosa. Consequently, the analysed trabeculae of adult animals were likely to have sensed less forces in response to mechanical loading as those trabeculae mainly undergo remodelling. Therefore, within tibia of young growing rats, bone formation rate measured within the secondary spongiosa may increase more quickly in response to mechanical loading as compared to that within tibia of adult animals. As osteoporosis is mainly a problem at higher age, we were interested in the effects of training on bone adaptation in adults. To this end, we chose to study adult rats. Comparison of our data with those of young growing animal suggests that low-impact exercise, such as endurance running exercise for approximately two months, seems to be particularly effective in inducing an increase in trabecular bone formation in very young growing animals and less in adults. In adult animals, another loading regime and/or a very long duration of the training period are likely required to enhance bone formation in trabecular bone.

Does endurance training on top of peak power training enhance the osteogenic response? As we expected that PT and ET had distinct effects on bone formation and resorption, we hypothesised that endurance training on top of peak power training would enhance the osteogenic response more than one type of training only. Although mRNA expression levels of OPG were higher in PET than those in PT, the combination of peak power and endurance training showed no differences in mRNA expression levels of any analysed gene compared with those in ET or controls. This indicates that the reduced osteo-anabolic response observed after PT was counteracted by additional endurance training. Despite the fact that in the PET group the mean mRNA expression levels of genes involved in bone formation were similar to those in the ET and control group, the variation of the mRNA expression levels of these genes was substantially higher, which could not be explained by the variation in mRNA ex-
pression levels of the housekeeping gene. Half of the rats of the PET group showed mRNA levels which were 1.5-2 fold higher than those in control and the other training groups. The observed high variations in particular genes suggest that in half of the rats of the PET group, daily combined training for 6 weeks had a cumulative positive effect on mRNA levels of genes involved in bone formation. Possibly, among the rats of the PET group, there were responders and non-responders to combined peak power and endurance training. In humans, such subject-to-subject variability in the adaptive response to training is well known [182]. Also among rats, differences in training-induced adaptation have been reported [184] as well as differences in running behaviour [147]. These data suggest that for some rats, combined training may have been a very effective training strategy to enhance the osteogenic response. As individual differences in training response may also exist in human subjects, such variation should be taken into account when developing training programs.

In osteoporotic older people, high-impact loading is generally dissuaded by physicians, as this increases the risk of (vertebral) fractures [129]. Additional endurance training on top of peak power training seems to be a useful training strategy to increase bone formation with a minimal amount of high-impact loading. Recently, a meta-analysis on training effects on postmenopausal bone loss showed beneficial effects of the combination of different training regimes on bone mineral density which could not be shown after high-impact training alone [118]. Although after PET we could not show a significant increase in bone formation, endurance training on top of peak power training seems to have prolonged the transcriptional response of osteo-anabolic factors within bone cells compared to those for PT or ET alone. This suggests that combined training could in the long-term result in an enhanced osteogenic response. Taken together, the results of this study and the reported effects in humans suggest that a combination of peak power and endurance training may be an appropriate strategy to increase bone formation. Further investigation is required to investigate the effects of intensity, duration and rest periods between training sessions in order to optimise training strategies. If the amount of high-impact loading during the training can be reduced and still increase the osteogenic response, this training strategy would be beneficial for osteoporotic older people to increase bone mass with a minimal risk for osteoporotic fractures.
In summary, 6 weeks of peak power training, endurance training or a combination thereof in rats does not change the rate in bone turnover in trabecular bone. Trabecular bone is more likely to increase in very young growing animals in response to such physical stimuli, whereas other types of mechanical loading and/or much longer training periods may be required to increase bone formation in adult animals. However, due to training, mRNA levels of osteo-anabolic and catabolic factors changed in cortical bone, suggesting effects prior to changes in bone turnover. In contrast to the expected increase in bone formation after PT, PT reduced the expression levels of osteo-anabolic factors, suggesting that if PT has an effect on the rate of bone turnover this will be an osteo-catabolic effect. The reduced OPG mRNA expression after PT was counteracted when additional endurance training was performed. The combination of peak power and endurance training with rest periods between the training sessions may prolong the transcriptional response of genes involved in bone turnover and may in the longer term enhance osteogenesis.

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