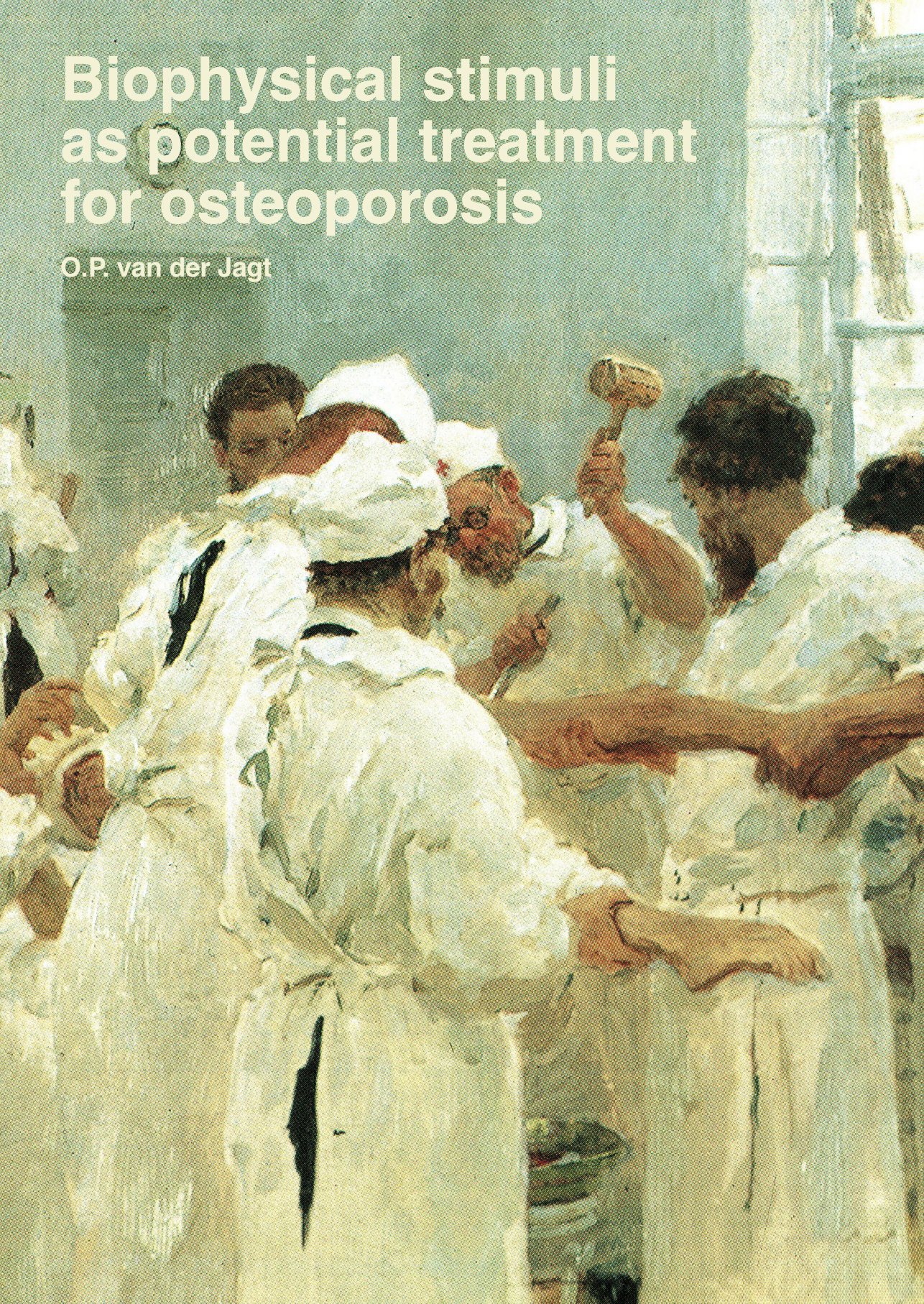
Biophysical stimuli as potential treatment for osteoporosis

O.P. van der Jagt



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Biophysical stimuli as potential treatment for osteoporosis

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00 Content

01
Introduction

INTRODUCTION

Osteoporosis is a disease characterized by diminished bone mass and deterioration of the bone microarchitecture leading to a higher susceptibility for fractures. The best known 'osteoporotic fractures' are those of the hip and vertebrae because these fractures have the most detrimental effects[1, 2]. However, other fragility fractures of the distal radius, humerus, ankle, pelvis, clavicula, and ribs account for 67% of all osteoporotic fractures and also significantly affect a patient's wellbeing and performance, although generally for a shorter period of time[3-5].

The incidence of osteoporotic fractures in the Netherlands is comparable to that in other West-European countries, which is higher than that in the USA for other (yet undetermined) reasons, that are most likely attributed to lifestyle factors[4, 6, 7]. The incidence varies widely between sexes, ages, races and the existence of other risk factors such as glucocorticoid use, low body mass index, smoking, rheumatoid arthritis and previous fractures[8, 9]. In the Netherlands, two-thirds of the patients aged 55 years and older with a hip fracture are women. The incidence of a hip fracture strongly depends on age. In women aged 65-69 years the incidence is 1.6 per 1000, whereas in women aged 75-79 years it is 7.1 per 1000[10]. In contrast to hip fractures, the incidence of wrist fractures does not rise with age. The incidence of wrist fractures in women older than 55 years is 6 in 1000, leading to more than 12,000 wrist fractures in women in the Netherlands annually[11]. For vertebral fractures it is much harder to present incidence data because many vertebral fractures occur without any trauma, and at the moment of the fracture many patients do not seek medical help[12].

It is known that both hip and vertebral fractures lead to an increased mortality risk[1, 13]. Mortality rates after hip fracture are reported to range from 8.4% to 36% during the first year, with the highest index for elderly men[14, 15]. However, also major fractures (including those of the proximal humerus, distal femur and proximal tibia) in patients aged 60 years and older, and minor fractures (including wrist and ankle fractures) in patients aged 75 years and older, are also associated with increased mortality[1, 16, 17].

It is not exactly known why fractures are related to increased mortality. No difference in cause of death can be found between patients with or without a history of fractures[16]. It is thought that in frail patients having multiple pre-existing co-morbidities, a hip fracture is just another late-life illness leading to death due to subsequent complications, primarily from infections (pneumonia) and cardiac illnesses (arrhythmias)[18, 19]. However, increased mortality rates are also seen in patients with osteoporotic fractures who are only 60 years old and not frail. In

addition, increased mortality risk lasts for many years, making it unlikely that only direct complications are related to the cause of death[16, 20, 21].

The consequence of having an osteoporotic fracture for the individual patient is much broader. Again, the most devastating and prolonged consequences are seen in hip fractures, in which many patients cannot return to home and need long-term institutionalization (14-55%), are unable to walk independently (60%), and/or are restricted in activities of daily living (ADL) (80%)[2, 22, 23]. Surprisingly, much less is known about the effects on general wellbeing and performance of other (minor) fragility fractures[5, 24-26].

In order to reduce osteoporosis-related mortality and morbidity, fracture prevention is the primary goal in osteoporosis treatment. Bisphosphonates, in combination with lifestyle modifications (ceasing to smoke, reducing alcohol consumption and increasing physical activity) and supplementation of calcium and vitamin D, is today's standard treatment of osteoporosis[27]. Alternative drugs such as strontium renalate, raloxifen, teriparatide and denosumab are also registered for the treatment of osteoporosis[28].

Bisphosphonates reduce excessive bone remodeling to retain bone mass and are known to reduce the incidence of vertebral and hip fractures[29, 30]. Side-effects include gastro-intestinal complaints, musculoskeletal pain and osteonecrosis of the jaw, of which the latter is relatively rare[31]. It has recently been shown that blocking bone resorption and subsequently bone remodeling leads to an increase in atypical fractures of the femur [32-34]. Characteristically a subtrochanteric stress fracture occurs, which leads to pain or a complete fracture. However, the incidence of these bisphosphonate-related fractures is low and the beneficial effects on osteoporotic fracture reduction seem to outweigh this complication[32-34].

The major disadvantage of bisphosphonates is that it primarily reduces further bone loss and that it is not anabolic. Currently the only registered anabolic agent is teriparatide (a recombinant homologue of parathormone) which, compared with bisphosphonates, is not superior in fracture reduction[9, 27]. Other targeting strategies (blocking cathepsine K or sclerostin) are currently being evaluated and may increase bone mass without blocking bone formation[9].

As an alternative to pharmaceutical treatments, in small animal models we investigated the effects of several biophysical stimuli on bone microarchitecture. We examined whether whole body vibrations (WBV), pulsed electromagnetic fields (PEMF) and extracorporeal shock waves (ESW) could influence bone architecture, in order to determine their possible use in the treatment of osteoporosis.

Outline of the thesis

The first biophysical stimulus we examined was treatment with whole body vibrations (WBV) in osteoporosis. It is well known that mechanical loading affects bone microarchitecture. In the 19th century Julius Wolff described the typical architectural lining of the trabeculae in the proximal femur, in which the orientation of the trabeculae is formed along the mechanical loading axes[35]. Other examples of bone adaptation to mechanical loading are: tennis players who have a higher bone mass in their dominant arm compared to their less mechanically loaded contra-lateral arm and, conversely, the fact that bone mass decreases during space travel when bone is not mechanically loaded[36, 37].

Much research has been dedicated to exploring the effect of mechanical loading and bone architecture. Many of these animal experiments focused on supraphysiological loading of the tibia or ulna[38]. Indeed, it was shown that high strains resulted in increased bone volume. However, extrapolation of these experiments (in which high strains are applied to one long bone) to the clinical situation is very difficult. To overcome this, WBV was introduced. In a landmark article by Rubin et al., in which the hind legs of sheep received vertical vibrations for 30 min per day, it was shown that this therapy resulted in an increase in trabecular bone volume after 1 year of treatment[39]. In **Chapter 2** we present our experiments in which we examined the effect of WBV on bone microarchitecture. Various WBV protocols that differ in frequency, acceleration and rest periods were tested in ovariectomized rats, and changes in bone microarchitecture were analyzed using *in vivo* microCT scanning.

The second biophysical stimulus we examined is pulsed electromagnetic fields (PEMF). In the 1950s it was shown that, during axial loading of bone, an electrical current is generated[40]. This led to the hypothesis that an electrical current applied to bone, can affect bone formation. Since then, studies have investigated bone formation after electrical stimulation with direct implantation of anodes and cathodes, especially in experimental models for nonunions[41, 42]. Although some positive results were achieved, clinical application of this invasive approach proved to be difficult. Alternatively, non-invasive stimulation with electromagnetic fields was developed by producing an electrical current through a helical circuit. Alternating the direction of this current results in PEMF.

The effects of PEMF have been examined extensively *in vitro* and in animal studies. Cellular effects suggest that PEMF should affect bone remodeling and thereby promote fracture healing or bone adaptation[43-45]. However, no positive effect of PEMF in the treatment of nonunions has been demonstrated[46]. Observational studies indicate that PEMF might enhance fracture repair in nonunions, but the quality of these studies is poor and no well-designed randomized controlled trials exist

to support this data[47-51]. Since some groups believe in the benefit of PEMF, it has also been examined in relation to osteoporosis. Strong positive effects of PEMF on osteoporosis have been shown in both clinical and experimental studies[44, 52, 53]. However, due to several methodological flaws with these earlier studies, we examined the effects of local and systemic PEMF on osteoporosis in a rat model. These experiments are outlined in **Chapters 3 and 4**, respectively.

The third biophysical stimulus examined is the application of extracorporeal shock waves (ESW). Shock waves are acoustical pulses that are characterized by a high amplitude (≥ 120 MPa) and a short rise time (≤ 10 ns), and are followed by a longer low-magnitude negative wave (≤ 10 MPa)[54]. Although there are many devices that produce 'shock waves', they differ in their method of wave generation. Pneumatic, electro-magnetic and electro-hydraulic designs are commercially available and result in shock waves with very different characteristics. Of these, electro-hydraulically generated shock waves are the most powerful. The disadvantage of shock waves, especially if they are generated electro-hydraulically, is that treatment is rather painful, such that they cannot be applied without local or general anesthesia. Moreover, the effects of the different types of shock waves cannot be interpolated, and most devices used by physiotherapists and orthopedic surgeons are pneumatically-generated shock waves which have much lower amplitudes. ESW are widely used to disintegrate kidney stones. There is some anecdotal evidence from physicians that there is thickening of the iliac wing on control X-rays after ESW treatment.

In orthopedics, shock waves are used in a variety of musculoskeletal disorders like nonunions and delayed unions, diaphyseal fractures, stress fractures, osteonecrosis of the hip, Achilles tendinopathy, calcifying tendinitis and fasciitis plantaris[55-59]. The effects of ESW in osteoporosis have not yet been explored. Animal studies have shown that extracorporeal shock wave therapy (ESWT) leads to an increased differentiation of bone marrow stem cells towards osteoprogenitor cells, and that several growth factors that are important for bone regeneration, including VEGF, TGF-beta 1 and several BMPs, were upregulated after ESWT [60-63]. For these reasons we were also interested in investigating ESWT for treatment of osteoporosis.

Until now ESWT for musculoskeletal disorders is applied with focused character, in which the waves converge in a focal point. For the prevention of fractures in osteoporosis a focused character is not preferable because large skeletal regions have to be treated, hence the proximal femur, forearm, ankle, vertebrae, etc. For the application of ESW in dermatologic conditions, generators that produce unfocused shock waves have been developed. With these devices a parallel bundle

is produced, enabling a homogenous treatment of larger areas. This gives the opportunity to treat large skeletal sites.

In three studies we explored the effects of unfocused extracorporeal shock waves (UESW) in osteoporosis by examining the effect on bone microarchitecture, bone remodeling, biomechanical properties and histological appearance. Several treatment protocols in multiple osteoporosis models and non-osteoporosis models were examined. These results are presented in **Chapters 5 to 7**.

Finally, a general discussion on the results of the work presented in this thesis is provided in **Chapter 8**.

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02

Low-magnitude whole body vibration does not affect bone mass but does affect weight in ovariectomized rats

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ABSTRACT

Mechanical loading has stimulating effects on bone architecture, which can potentially be used as a therapy for osteoporosis. We investigated the skeletal changes in the tibia of ovariectomized rats during treatment with whole body vibration (WBV). Different low-magnitude WBV treatment protocols were tested in a pilot experiment using ovariectomized rats with loading schemes of 2 x 8 min/day, 5 days/ week (*n*=2 rats per protocol). Bone volume and architecture were evaluated during a 10 week follow-up using *in vivo* microCT scanning. The loading protocol in which a 45 Hz sine wave was applied at 2 Hz with an acceleration of 0.5*g* showed an anabolic effect on bone and was therefore further analyzed in two groups of animals (*n*=6 each group) with WBV starting directly after or 3 weeks after ovariectomy and compared to a control (non-WBV) group at 0, 3, 6 and 10 weeks' follow-up. In the follow-up experiment the WBV stimulus did not significantly affect trabecular volume fraction or cortical bone volume in any of the treatment groups during the 10 week follow-up. WBV did reduce weight gain that was induced as a consequence of ovariectomy. We could not demonstrate any significant effects of WBV on bone loss as a consequence of ovariectomy in rats; however, the weight gain that normally results after ovariectomy was partly prevented. Treatment with WBV was not able to prevent bone loss during induced osteoporosis.

INTRODUCTION

Mechanical loading has potentially stimulating anabolic effects on bone architecture, which can be used as a therapy for osteoporosis, a disease characterized by low bone mass. Currently the standard treatment focuses on bone resorption inhibitors, which increase bone mineral density (BMD) and subsequently lower the risk for hip and vertebral fractures[1]; however, these pharmaceuticals require a frequent and (probably) lifelong intake with potential side-effects.

Whole body vibration (WBV) can be performed at different settings. In studies examining the effects on bone it is generally applied in a low-magnitude mode, resulting in small microstrains (<10 microstrains). The osteogenic potential of WBV was demonstrated by a study in which the hind legs of sheep were subjected to mechanical vibration for 1 year (30 Hz and an acceleration of 0.3*g*, for 20 min/day) resulting in a 32% higher trabecular bone volume fraction compared to non-treated controls[2]. In rodents it was found that osteoclastic activity decreased and bone formation rate increased after low-magnitude WBV[3, 4].

Few animal studies have examined the effect of WBV on osteoporosis specifically. In a longitudinal study it was found that ovariectomized rats that received WBV had a higher BMD in the femur and the tibia compared to nonvibrated controls after 5 weeks of WBV but not after 12 weeks of WBV[5]. Oxlund et al.[6] found that WBV at formation rate, but vibrations of 17 Hz, 0.5*g* and 30 Hz, 1.5 *g* did not result in significant differences from control animals. In another study bone formation rates were largely affected when WBV at 90 Hz, 0.15 *g* was applied but not at 45 Hz[7]. These altered bone formation rates at 90 Hz did result in a higher volume fraction in the epiphysis, but not in the metaphysis.

In studies using ulnar or tibial bending it was shown that inclusion of rest periods between loading cycles of seconds, hours and also weeks further stimulated the bone formation rate[8-10]. It was also reported that when a noise signal that is on its own not osteogenic, is added to a low-magnitude vibration, bone formation rate increased almost fourfold[11]. These studies suggest that a continuous stimulus might decrease the mechanosensitivity of the skeleton and that that nonlinear characteristics in the loading regime enhance the potential anabolic effects of WBV.

To further elucidate the anabolic effect of whole body mechanical vibration on osteoporosis we investigated the skeletal changes in the tibia of ovariectomized rats during treatment with WBV. *In vivo* microcomputed tomography (microCT) scanning was used to evaluate skeletal changes. First we explored different WBV characteristics in a few animals to identify potential osteogenic stimuli. Subsequently we examined a successful stimulus in a higher number of animals to confirm the osteogenic effect.

Materials and methods

In total 38 female Wistar rats were purchased (Harlan, Boxmeer, The Netherlands). The animals were housed in pairs in the institute's animal facility with 12 h light/dark regimen, in the presence of standard food pellets (CRM (P) from Special Diets Services, UK, with a gross energy of 15.01 MJ/kg, containing 0.83% calcium and 3077.42 IU/kg vitamin D) and water ad libitum. The study protocols were approved by the local Animal Experiments Committee (EUR 536 and 940) and were in accordance with Dutch law on animal experimentation.

To simulate osteoporosis a bilateral ovariectomy (OVX) was performed at 20 weeks of age. This was performed under sterile conditions using gas anesthesia (oxygen with 3% isoflurane; Rhodia Organique Fine Ltd., Bristol, UK). Analgesics were given for 2 days: 0.05 mg/kg/12 h buprenorfine (Schering-Plough, Kenilworth, NJ, USA). During treatment with WBV the animals were moved from their housing cage to the cage that was connected to an oscillator. The oscillator was controlled with a custom-made computer program using Labview software (National Instruments, Austin, TX, USA). Using an accelerometer the accelerations of the cage were registered in real time in order to monitor the applied mechanical stimulus.

In the first phase of the study 9 signals were investigated with different characteristics (Table 1; Fig. 1). The effect of each signal was investigated in 2 animals per signal during a follow-up period of 10 weeks and compared to a non-vibration control group. The animals were subjected to WBV twice a day for 8 min. Each stimulus consisted of a vertical acceleration (sinusoid waveform) with a superimposed noise signal (low-amplitude Gaussian quasi white noise (0–50 Hz)[11]. The examined mechanical vibrations varied in frequencies, accelerations and rest periods between the loading cycles as summarized in Table 1 and Fig. 1. WBV was started 2 days after OVX and *in vivo* microCT scans were performed at 0, 3 and 10 weeks after OVX.

Table 1. Overview experimental groups

no.	frequency	acceleration	applied
1	2 Hz	0.5g	2x/day, 5d/wk, every wk
2	2 Hz	2g	2x/day, 5d/wk, every wk
3	45 Hz	0.5g	2x/day, 5d/wk, every wk
4	45 Hz	2g	2x/day, 5d/wk, every wk
5	45-90 Hz noise	0.5g	2x/day, 5d/wk, every wk
6	2 Hz	0.5g	2x/day, 3d/wk, every wk
7	2 Hz	0.5g	2x/day, 5d/wk, 1 wk applied, 1 wk rest
8	2 Hz	0.5g	2x/day, 5d/wk, 3 wks applied/3wks rest
9	45Hz given at 2Hz	0.5g	2x/day, 5d/wk, every wk
control	sham	sham	2x/day, 5d/wk, every wk

Different characteristics of the nine protocols examined in the first phase of the experiment. The basic sinusoid waveform was superimposed by 0-50 Hz very low amplitude noise.

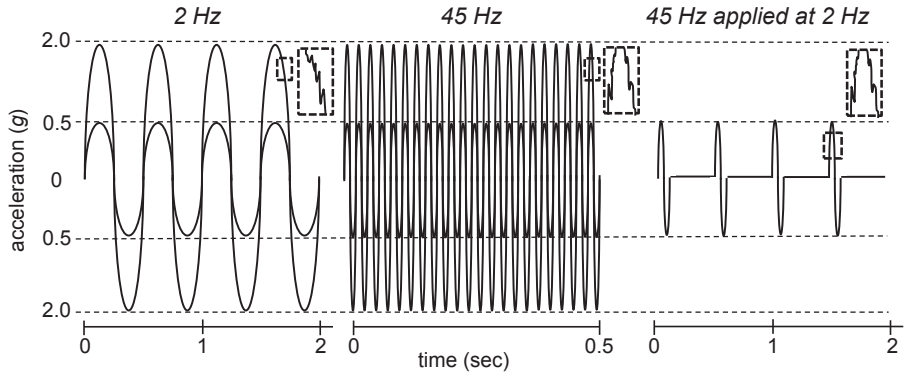

Fig. 1: Representation of different vibrations at 2, 45 and a 45 Hz given at 2 Hz, both 0.5 and 2.0 g acceleration. All vibrations were superimposed with 0-50 Hz noise signal (see inset).

In this pilot phase one stimulus showed a positive effect and was analyzed in more detail in the follow-up experiment. This stimulus was a 45 Hz sinusoid waveform superimposed on 2 Hz, 0.5*g* with 10 s on/off regime. Two groups of 6 animals were treated twice a day for 8 min, 5 days per week. In one group WBV started immediately (day 2) after OVX and in the second group WBV started 3 weeks after OVX. A third group (*n*=6) did not receive mechanical vibrations and served as a control. Bone microarchitecture was analyzed at 0, 3, 6 and 10 weeks after OVX.

To evaluate the bone changes in the proximal tibia *in vivo* microCT scans were performed. Under gas anesthesia the hind leg of the rat, from the distal femur until the tibial diaphysis, was scanned with an 18-micron voxel size using an *in vivo* microCT scanner (Skyscan 1076 microtomograph, Kontich, Belgium) at a voltage

of 60 kV, a current of 167 µA and a 0.5 mm aluminum filter, over 196˚ with a rotation step of 1 , taking 8 min per scan. Using NRecon (NRecon software version 1.5, Skyscan) three-dimensional (3D) reconstructions of two regions of interest were made, one at the proximal metaphysis which mainly contains cancellous bone and another at the middiaphysis, which contains mainly cortical bone (Fig. 2). The reconstruction of the proximal metaphysis was selected manually starting just distally of the epiphysis and continuing distally until 3.6 mm. The reconstruction of the diaphysis was defined by a region of 3.6 mm starting 9 mm distally from the epiphysis. Bony and non-bony structures were discriminated using a local threshold algorithm (software freely available)[12] resulting in binary datasets[13]. Cortical and trabecular bone were automatically separated using in-house software. Trabecular architecture of the proximal metaphysis was characterized by determining trabecular bone volume fraction (BV/TV), connectivity density (Conn/TV), structure model index (SMI) and mean 3D trabecular thickness (TbTh). Cortical architecture was assessed in the diaphysis and was characterized by cortical volume (CtV) and cortical thickness (CtTh).

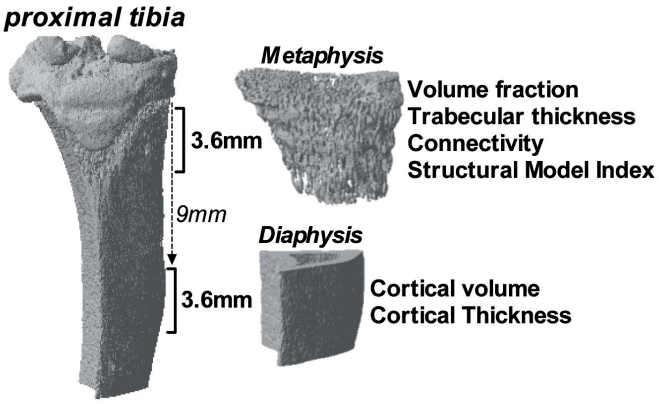

Fig. 2 MicroCT scanning. 3D reconstruction of a microCT scan of the proximal tibia and proximal fibula. The analyzed regions of interest are indicated.

For the follow-up experiment the differences between the means of each vibration group and the means of the control group were statically analyzed using ANOVA (GraphPad Software, San Diego, CA, USA).

RESULTS

Experiment 1: pilot study

At the start of the experiment at 0 weeks the BV/TV was 34.4% (SD 4.4), TbTh was 117.7 μm (SD 7.8), Conn/TV was 94.3 (SD 17.3), CtV was 15.2 mm^3 (SD 1.0), and CtTh was 644.9 μm (SD 40.6).

During the experimental period the BV/TV declined in control animals to 23.2% (SD 3.7) at 3 weeks and to 14.2% (SD 3.7) at 10 weeks after OVX (Fig. 3a). Compared to the control group only one treatment showed a discriminative pattern of bone loss between 3 and 10 weeks (Fig. 3a). This specific treatment consisted of mechanical vibration of 45 Hz given at 2 Hz with an acceleration of 0.5*g* (protocol no. 9, Table 1 and Fig. 1). Although both animals seemed to respond to this stimulus the magnitude was different, given that one animal showed anabolic effects and the other a subtle decrease in bone loss compared to controls (Table 2). Neither the cortical changes of this treatment group nor of the other treatment groups were different from control animals not receiving mechanical vibration (Fig. 3b; Table 2).

During the experimental period the average weight gain during the 10 week follow-up was not different between vibrated and control rats, with 68.8 g (SD 17.0) and 71.0 g (SD 4.2), respectively ($p< 0.05$).

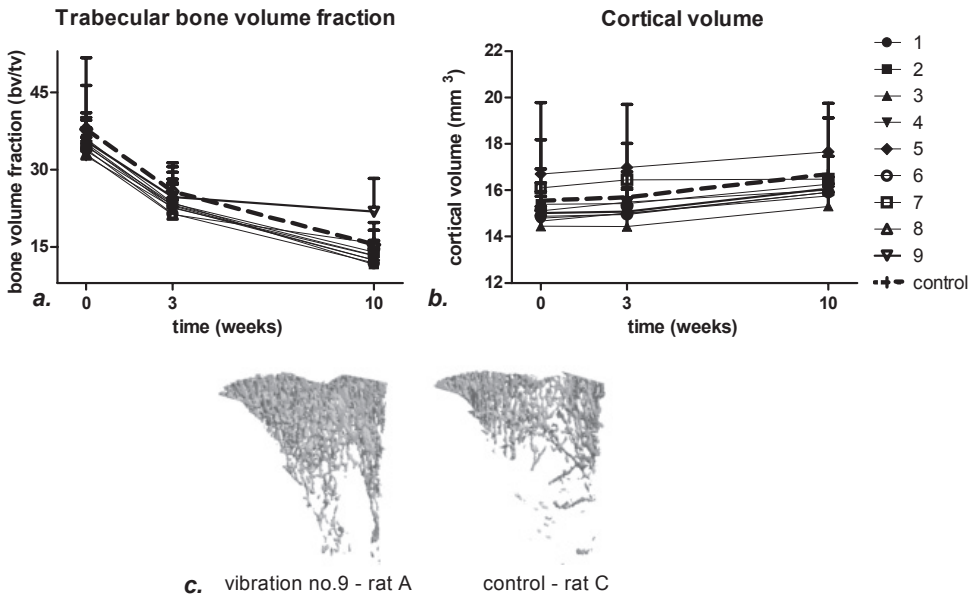

Fig. 3 Experiment 1: pilot study. Trabecular volume fraction (a) and cortical volume (b) in the proximal tibia of ovariectomized rats subjected to (sham)-WBV (mean with SD). Section of a 3D reconstruction of a control rat and a rat that received protocol no. 9 (c).

Table 2 Bone parameters in controls and 45Hz at 2Hz 0.5 g vibration

	week 3		week 10	
	control	mechanical vibration	control	mechanical vibration
BV/TV (%)	25.8 (4.8)	24.7 (0.2)	15.4 (4.3)	21.8 (6.4)
Trabecular Thickness (µm)	108.2 (2.0)	105.1 (1.0)	112.1 (0.8)	114.6* (1.4)
SMI	2.1 (0.3)	2.2 (0.01)	2.6 (0.3)	2.2 (0.3)
Connectivity density	64.4 (23.1)	63.3 (1.8)	17.7 (13.4)	42.0 (24.4)
Cortical Volume (µm³)	15.7 (2.3)	15.1 (1.0)	16.7 (2.4)	16.0 (0.2)
Cortical Thickness (µm)	701.6 (34.2)	663.9 (37.0)	747 (38.5)	699 (8.9)

Bone changes in the tibia of non-vibrated control rats and rats that received a 45Hz vibration given at 2Hz in the pilot study. WBV was started 2 days after OVX. Mean (SD), n=2 per group. * indicates $p<0.05$ compared to control.

Experiment 2: follow-up study

The BV/TV of control animals declined to 23.7% (SD 3.6) at 3 weeks, 16.3% (SD 2.6) at 6 weeks, and 12.0% (SD 2.4) at 10 weeks after OVX (Fig. 4). The CtV increased from 15.7 mm³ (SD 0.8) to 16.4 (SD 1.1), 17.1 (SD 1.0), and 17.2 mm³ (SD 1.1) at 3, 6 and 10 weeks, respectively.

There was no difference between the animals that received WBV immediately after OVX and the control animals. In contrast to the exploratory phase, none of the six animals responded to the stimulus. Also, when 3 weeks of bone loss was allowed before the start of treatment, no effect from WBV was observed at an acceleration of 0.5*g* (Fig. 4).

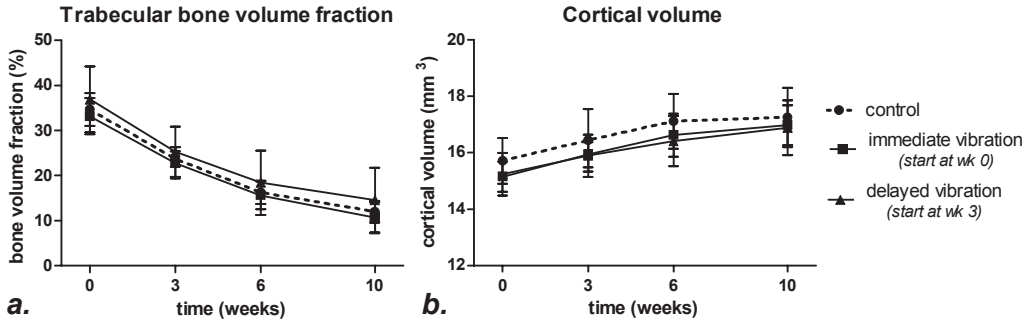

Fig. 4 Experiment 2: follow-up study. Trabecular volume fraction (a) and cortical volume (b) in the proximal tibia of ovariectomized rats subjected to a 45 Hz vibration given at 2 Hz, starting at week 0 or at week 3 after OVX (mean with SD).

As a consequence of OVX the animals gained weight during follow-up; however, there was a difference between animals that received WBV and controls (Fig. 5a). Between 0 and 3 weeks no difference was observed, but animals that started WBV

at 3 weeks lost weight between 3 and 6 weeks in contrast to controls, with -1.3 g (SD 5.2) and 9.3 g (SD 4.7), respectively ($p < 0.01$). Between 6 and 10 weeks no significant difference was observed; however, the weight gain during the 10 week follow-up was different between controls and both WBV groups (Fig. 5b). The average weight gain of control rats was 88.5 g (SD 13.2) compared to 70.3 g (SD 6.4) and 62.7 g (SD 21.9) in the immediate vibration, and delayed vibration group ($p = 0.01$ and $p = 0.03$, respectively).

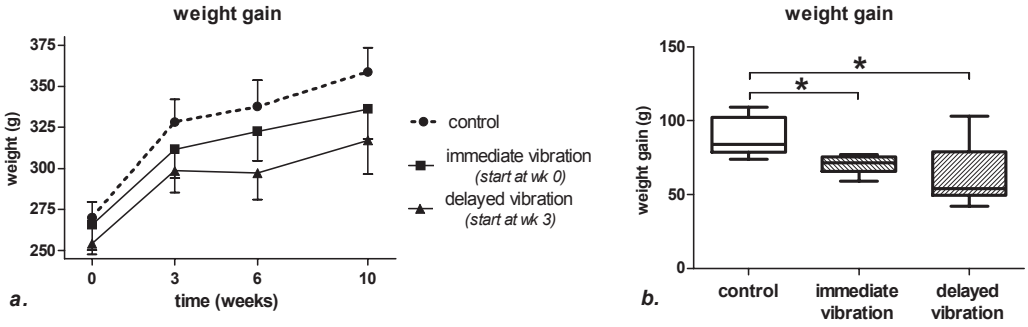

Fig. 5 Weight gain. Weight during follow-up (a) and 5–95% whisker boxplot (of weight gain during the 10 week follow-up, Asterisk (*) indicates p>0.05 (b). The boxes represent the 5 to 95% confidence intervals, the median is shown by the horizontal lines within the boxes, and the I bars represent the minimum and maximum values.

DISCUSSION

In the current study the effects of WBV were examined using *in vivo* microCT scanning allowing longitudinal follow-up of the bone microarchitecture in the proximal tibia of individual rats. In a pilot study, in which many different vibrations were examined, one stimulus affected the trabecular bone architecture; however, this effect could not be confirmed in the follow-up experiment. This data might suggest that some individual rats might be sensitive to a specific vibration, but it does not support the notion that WBV improves bone architecture as a benefit for osteoporotic patients in general. However, WBV clearly reduced OVX-induced weight gain.

To date, positive findings of the effect of WBV on bone morphology are scarce. Rubin et al. found that sheep that received WBV for 20 min/day had 32% higher trabecular bone volume fraction than non-vibrated controls. These impressive results have never been reproduced in studies using rodents[2]. In mice it was shown that dynamic histomorphometric parameters like bone formation rate and osteoclastic

resorption were clearly affected by WBV (45 Hz, 0.3*g*, 15 min/day, 5 days/week), but cortical bone volume showed only a small increase and trabecular bone volume fraction was not affected[3, 4]. In rats it was also observed that low-magnitude WBV (45 or 90 Hz, 0.15*g*, 10 min/day, 5 days/week) led to an increased bone formation rate in the metaphysis, while trabecular bone volume fraction did not increase[7].

Two other papers also report no effect of low-magnitude high-frequency WBV on microarchitectural parameters[14, 15]. One study examined the effect of a 90 Hz sine wave with a maximum acceleration of 0.3*g*, applied 5 days/week in ovariectomized and sham-ovariectomized rats[14]. The other study used mice and was applied to a 90 Hz sine wave with a maximum acceleration of 0.3 or 1.0*g*[15]. In contrast to these studies another study in which a 90 Hz WBV with an amplitude of 0.5 mm was applied to sham-ovariectomized rats did result in increased trabecular and cortical structural parameters[16].

Use of *in vivo* microCT scanning allows the detection of very small changes, because longitudinal data are collected. In our pilot experiment one stimulus seemed to reduce the OVX-induced bone loss; however, this effect could not be reproduced in our follow-up experiment. Given the data of the pilot study and the use of 6 animals, we would have been able to detect the large pilot effect (>40%) with a power of close to 1. Given the more robust data from the full-size experiment we would have been able to detect an effect of 11% (which is 2.6% difference in BV/TV) at a power of 0.8 and amplitude of 0.05, which is far less than the effect found during the pilot test (>40%). Furthermore, the effect found at the pilot test was mainly caused by one animal that hardly lost any bone after treatment, suggesting that the effectiveness of a stimulus could be animal dependent. However, in our follow-up experiment none of the animals showed a reduction in bone loss that was different from the variation found in the control group.

To date, WBV in humans has shown contradictory effects. In a prospective study by Verschueren *et al.* it was shown that the BMD in the hip of postmenopausal women who received WBV for 24 weeks (30–40 Hz, 2.3–5.1*g* for a maximum of 30 min/day) was significantly higher than non-treated controls[17]. However, in another study with postmenopausal women, 6 months of WBV (30–40 Hz, 1.6–2.2*g*, 12 min three times per week) did not influence BMD in the hip[18]. In a study by Rubin *et al*. in which postmenopausal women were included and in a study by Torvinen *et al*. of volunteers between 19 and 38 years, WBV did not affect the BMD at the hip, spine or distal radius after 12 and 8 months, respectively[19, 20]. WBV was also unable to enhance the BMD in the spine of osteoporotic patients treated with alendronate[21]. Bone serum markers were not influenced by WBV in any of these trials.

A shortcoming of the current paper is that no shamovariectomized animals were examined. There are some reports describing the interaction between the mechano-

sensitivity of the skeleton and the presence of estrogen and/ or estrogen-receptors[22, 23]. Although this view is under a lot of debate[24] the study would have been stronger if the effect of the different WBVs were also examined in non-ovariectomized rats. However, in a recent paper using sham-ovariectomized and ovariectomized rats with loaded and unloaded hind legs it was demonstrated that the skeletal changes induced by estrogen loss were at other skeletal regions than the changes induced by loading and that the effects on bone due to estrogen or loading are therefore different entities[23]. Another limitation of the current study is that the strains induced by the vibrations were not measured. Since the characteristics of the vibration were at the same order of magnitude as the vibrations applied by others, we believe that the strains will also be at the same order of magnitude (between 1 and 2 $\mu\varepsilon$)[7].

Since the effect of WBV on weight gain was unexpected in the current study we did not analyze which tissues were specifically affected; however, the effect of OVX on hyperphagia and subsequent gain in fat tissue has been extensively examined[25]. Currently, the effects of low-magnitude WBV on adipogenesis is receiving a lot of attention, since several reports have shown an inhibiting effect of low-magnitude WBV on adipogenesis and body fat accumulation[26-28]. These effects might be related to the differentiation of mesenchymal stem cells towards osteoblasts and adipocytes. It was found that the terminal differentiation of adipocyte progenitors is inhibited by loading, because PTH/PTHrP signalling pathways were affected[29]. This might also explain our findings of reduced weight gain in animals that received WBV.

In conclusion the current study does not support the use of WBV as therapy for osteoporosis, but it was clearly effective at reducing OVX-induced weight gain.

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03

Systemic treatment with pulsed electromagnetic fields do not affect bone microarchitecture in osteoporotic rats

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ABSTRACT

Pulsed electromagnetic fields (PEMF) are currently used in the treatment of spinal fusions and nonunions. There are indications that PEMF might also be effective in the treatment of osteoporosis. In this study we examined whether whole-body PEMF treatment affects the bone microarchitecture in an osteoporotic rat model. Twenty-week-old female rats were ovariectomized (*n*=20). Four different PEMF treatment protocols based on previous experimental studies and based on clinically used PEMF signals were examined (2 h/day, 5 days/week). A control group did not receive PEMF. At zero, three and six weeks cancellous and cortical bone architectural changes at the proximal tibia were evaluated using *in vivo* microCT-scanning. PEMF treatment did not induce any changes in cancellous or cortical bone compared to untreated controls. Although previous studies have shown strong effects of PEMF in osteoporosis we were unable to demonstrate this in any of the treatment protocols. Using *in vivo* microCT-scanning we were able to identify small bone changes in time. Subtle differences in the experimental setup might explain the differences in study outcomes in the literature. Since PEMF treatment is safe, future experimental studies on the effect of PEMF on bone can better be performed directly on humans, eliminating the potential translation issues between animals and humans. In this study we found no support for the use of PEMF in the treatment of osteoporosis.

INTRODUCTION

Osteoporosis is a disease characterised by progressive bone loss and deterioration of the microarchitecture leading to an increased fracture risk. Osteoporosis can have distinctive causes including lack of sex hormones, long-term use of glucocorticoids or disuse. Current standard therapy consists of reducing further bone loss using bisphosphonates[1]. The use of bisphosphonates is accompanied by potential side effects such as gastrointestinal complaints, osteonecrosis of the jaw and atypical femoral fractures[2]. As an alternative treatment biophysical stimuli have long been proposed. These might be cheaper and induce fewer side effects. Pulsed electromagnetic fields (PEMF) might be one such treatment and indeed there is some evidence that these positively influence bone mass.

The finding that electrical currents are induced during mechanical loading of bone has led to the development of PEMF[3, 4]. Much research has been done on the effects of PEMF on bone. *In vitro* studies do show that a variety of growth factors that are important in bone metabolism are affected, including bone morphogenetic protein 2 (BMP-2), transforming growth factor beta (TGF-β) and insulin-like growth factor II (IGF-II)[5-11]. Furthermore, PEMF result in the activation of extracellular signal-regulated kinase (ERK), mitogen-activated protein kinase (MAPK) and prostaglandin synthesis, which might also lead to stimulatory effects on bone[12-14]. Clinically PEMF are widely used for the treatment of nonunions, although they have never been proven to be effective in a prospective randomised controlled trial[15-22].

PEMF as treatment for osteoporosis have been studied before. In a clinical study it was shown that PEMF treatment of the wrist induced an increase in bone mineral density (BMD) in the distal radius of osteoporosis-prone women[23]. Re-examination 12 years later did show that these effects had lapsed[24]. Only a few animal studies have been published on the subject. In one study complete preventive effects of PEMF on trabecular bone loss in an ovariectomized rat model were demonstrated[12]. PEMF consisted of a single pulse wave form with a maximum of 8 G and were applied eight hours per day. In another study it was shown that PEMF led to trabecular thickening in ovariectomized rats when treated with pulse bursts with a maximum of 9.6 G for six hours per day[25]. In a third study electromagnetic fields (EMF) induced pronounced cortical bone formation[26]. EMF consisted of a sinusoid wave form with a maximum electromagnetic field of 10 G and were applied for four hours per day. One study using osteoporotic rats did not demonstrate a beneficial effect on bone mass when treated with PEMF with 15 G for 24 hours per day[27]. Furthermore, PEMF have also been shown to influence bone mass in disuse osteoporosis both clinically and experimentally[28-30].

Because treatment of osteoporosis with PEMF can have important consequences for today's standard treatment, we examined the effect of whole-body PEMF treatment on the bone microarchitecture in a rat model of osteoporosis. Cancellous and cortical bone changes in the proximal tibia were followed over time using *in vivo* microCT-scanning.

MATERIALS AND METHODS

Twenty female Wistar WU rats were obtained (Charles River, The Netherlands). All animals were housed in pairs in the institute's animal facility with a 12-hour light/dark regimen and received standard food pellets and water ad libitum. The study protocol was approved by the local Animal Experiments Committee (EUR 415) and was in accordance with Dutch law on animal experimentation.

At an age of 20 weeks a bilateral ovariectomy (OVX) was performed under sterile conditions to simulate osteoporosis. This was done under 3% isoflurane gas anaesthesia (Rhodia Organique Fine Ltd., Bristol, UK). Buprenorphine 0.05 mg/kg per 12 hours (Schering-Plough, Kenilworth, NJ, USA) was given for pain relief for three days post-operatively.

The second day after OVX, PEMF treatment was started for five days/week over a period of six weeks. During treatment the animals were placed in a cage (24×30×12 cm) in pairs, with food and water ad libitum. The cage was surrounded by two connected coils normally used in the treatment of nonunions of the femur (IMD, Uden, the Netherlands) (Fig. 1). The rats were divided into five groups of four animals/group. The sample size was chosen for logistical reasons and not on the basis of a power analysis. The group sizes were kept relatively small because *in vivo* microCT analysis can accurately measure small changes over time. A custom-made generator produced the electromagnetic signals (see details below). The first group served as controls and was placed in a cage covered with non-functioning coils for two hours per day.

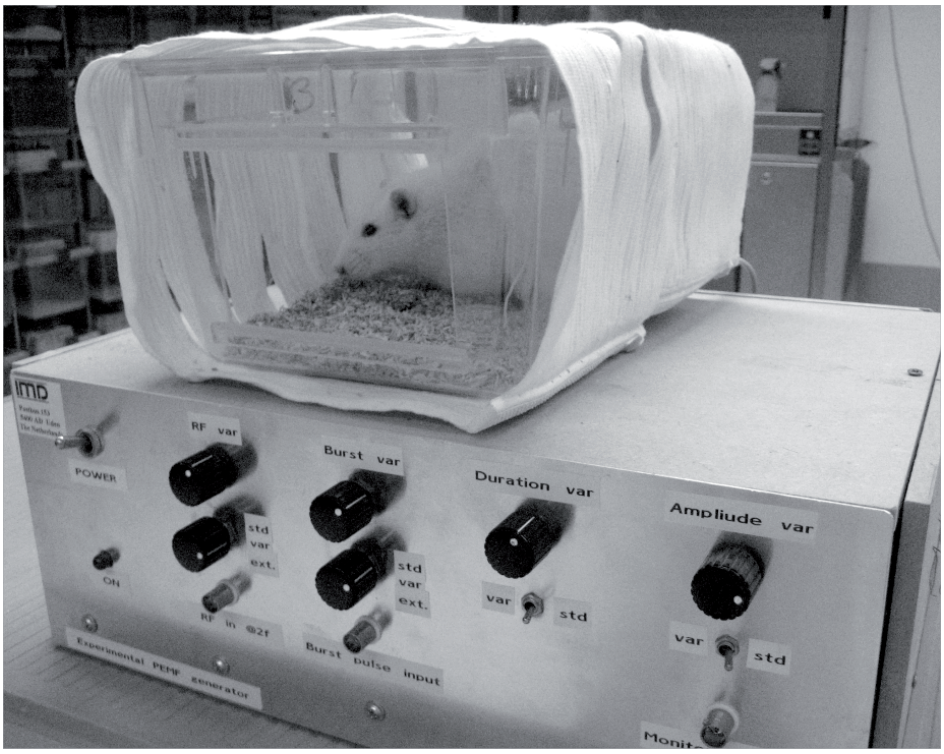

Fig. 1 Rat receiving systemic PEMF treatment. The cage is surrounded by coils. The custom-made generator can produce different electromagnetic signals.

The second group was treated with a commercially available PEMF device that is effective for the treatment of nonunions and is clinically used (Orthopulse®, IMD, Uden, The Netherlands)[31]. The device produces PEMF with a 1G electromagnetic field, consisting of 5-ms pulse bursts with 5-µs pulses, and the bursts repeat at 15 Hz. Rats were treated for two hours per day.

The treatment protocols of the third and fourth groups were designed to investigate whether a non-continuous PEMF stimulus would be more effective than a continuous stimulus, similar to studies investigating the effects of mechanical vibration on bone mass[32-34]. The third group received the same PEMF signal as the second group, but with a five minute on/off regimen; these animals were placed in the coil-covered cage for four hours per day to ensure that the total amount of PEMF time was the same in each animal. The fourth group also received the basic PEMF signal, but a custom-made amplifier added random noise of 50–150 kHz over the basic stimulus. Animals were treated with this latter signal for two hours per day.

A fifth group received a PEMF signal that was not characterised by pulse bursts as in the other treatment groups, but consisted of quasi-rectangular single pulses

of 1 G given at 7.5 Hz with a pulse duration of 0.3 ms. These animals were treated for two hours per day.

In vivo microCT scans were made at zero, three and six weeks after OVX. Using isoflurane (3%) rats were anaesthetised and the right hind leg was scanned (voltage of 60 kV, current of 167 µA, 0.5-mm aluminium filter, 196° with a rotation step of 1°) (SkyScan 1076 Microtomograph, Kontich, Belgium). Then 3D reconstructions with an isotropic voxel size of 18 µm were made of the proximal tibia and the middiaphysis (Nrecon software version 1.5, SkyScan) (Fig. 2). The proximal metaphysis mainly consists of cancellous bone. A length of 5.4 mm was selected of which the epiphysis was manually deselected. The mid-diaphysis consists of cortical bone and the region of interest was selected 9 mm distal from the epiphysis and continued to 3.6 mm more distally. Using a local threshold, bone was separated from nonosseous structures, which resulted in binary data sets[35]. After automatic separation between trabecular and cortical bone using in-house software bone parameters were determined (3Dcalculator, SkyScan)[36]. On the metaphyseal region of interest, volume fraction (BV/TV), connectivity density (Conn/ TV), structure model index (SMI), in which an index of 3 indicates the presence of rods and an index of 0 indicates the presence of plates, and 3D trabecular thickness (TbTh) were determined. On the diaphyseal region of interest, cortical volume (CtV) and cortical thickness (CtTh) were determined.

Differences between means of the different treatment groups were statistically analysed using one-way analysis of variance (ANOVA) with a Tukey's multiple comparison test for each time point separately (GraphPad Software, San Diego, CA, USA).

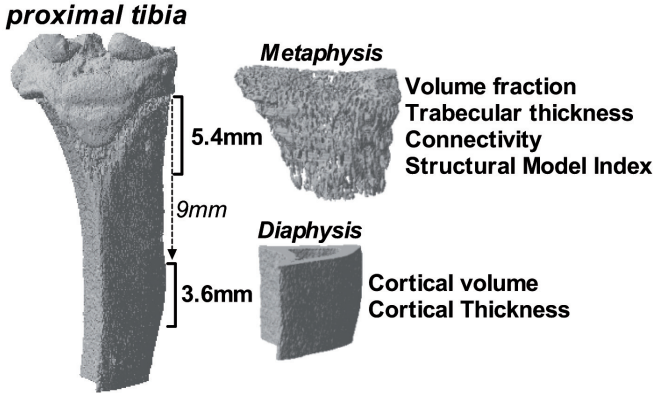

Fig. 2 Three-dimensional reconstruction of a microCT scan of the proximal tibia. The regions of interest in the metaphysis and diaphysis are indicated.

RESULTS

All surgical procedures were performed without complications. At the start of the study the average body weight of the rats was 224.6 g (SD 5.3). No period of weight loss was observed during the experimental period. The average weight gain was 57.6 g (SD 8.7) and there was no difference in weight gain between controls and any of the PEMF-treated groups.

In non-treated control animals BV/TV decreased from 31.7% (SD 4.8) at week zero to 24.2% (SD 4.1) and 19.7% (SD 3.5) at weeks three and six, respectively, as a consequence of OVX (Table 1). Morphometric parameters changed concomitantly, especially between weeks zero and three, with a decrease in mean TbTh, a decrease in Conn/TV and a structural change of trabeculae towards more rod-like structures, as apparent from an increase in SMI (Table 1). CtV increased from 12.3 (SD 0.23) mm^3 at week zero to 12.9 (SD 0.23) mm^3 and 13.3 (SD 0.43) mm^3 at weeks three and six, respectively. Mean CtTh also increased during this period.

None of the PEMF-treated groups showed a significant difference in BV/TV or CtV compared with the control group at any time point (Fig. 3). The morphometric parameters also showed no significant differences compared with control animals. There were no indications to speculate the presence of any non-significant trend in the follow-up outcome variables.

Table 1 Bone changes in the proximal tibia of ovariectomized non-treated control animals

	week 0	***week 3***	***week 6***
volume fraction (%)	31.7 (4.8)	24.2 (4.1)	19.7 (3.5)
trabecular thickness (μm)	127.3 (5.6)	125.0 (3.7)	118.9 (2.2)
connectivity density (/mm^3)	78.7 (14.5)	47.5 (15.6)	38.9 (12.9)
structure model index	1.7 (0.19)	2.1 (0.15)	2.2 (0.09)
cortical volume (mm^3)	12.3 (0.23)	12.9 (0.36)	13.3 (0.43)
cortical thickness (μm)	608.2 (9.4)	625.6 (4.0)	646.4 (15.4)

Mean values with standard deviations are given (n=4)

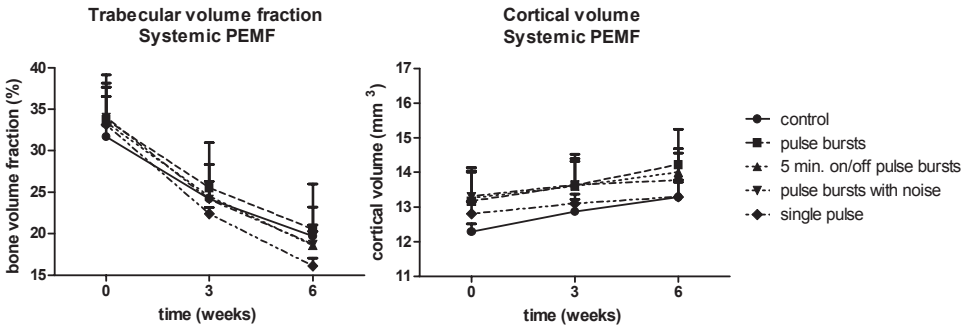

Fig. 3 Trabecular volume fraction and cortical volume in the tibia of ovariectomized rats that received PEMF treatment (n04) or received no treatment (controls, n04). Data are mean values and standard deviations

DISCUSSION

In this study we examined the effects of PEMF on bone changes in the cancellous and cortical bone during a six-week follow-up period. Although we used *in vivo* microCTscanning, which is a very sensitive analysis method, we were unable to reproduce the strong beneficial effects of PEMF on osteoporosis in ovariectomized rats as shown by others[12, 25, 26].

In this study four treatment protocols were used. Three of these protocols were based on a commercially available PEMF generator used for the treatment of nonunions. The fourth signal was developed as a single burst in order to be more comparable with the study of Chang and Chang[12]. In the study of Chang and Chang it was shown that PEMF prevented the trabecular bone loss induced by ovariectomy. Although one of the groups received PEMF with characteristics based on their signal, some differences in the experimental set-up still remain. Our signal had an electromagnetic current of 1 G instead of 4–8 G in the signal of Chang and Chang. Furthermore, they treated the rats for eight hours per day instead of two hours as used in our study. To examine whether longer exposure to PEMF would prevent further bone loss in our experimental set-up, we extended the experiment for an additional six weeks and treated the same animals for eight hours per day. However, trabecular bone loss was comparable to non-treated controls and no preventive effects of longer treatment could be found.

In a more recent article PEMF, consisting of pulse bursts with an electromagnetic current of 9.6 G, were applied to ovariectomized rats during day time or night time[25]. In both groups PEMF treatment resulted in an increase in BMD and increased trabecular bone mass compared to ovariectomized controls. It was shown that treatment during daytime was more efficient than an overnight treatment. No effect

on cortical bone was found. In another study in which ovariectomized rats were exposed to a 50-Hz sinusoidal waveform of 10 G an increase in cortical volume of 71% compared to non-treated controls was found[26]. One study has been published in which a negative finding is described. In that study 24 hours per day of systemic PEMF with a maximum magnetic field of 15 G did not result in an increased bone mineral content after eight months of treatment[27].

Apart from differences in the characteristics of PEMF treatment used in each study, the ages of the animals also vary. In the studies that did find a positive effect of PEMF, eight to 13 week-old rats were used[12, 25, 26]. In our study 20 week-old rats were used and in the study of Takayama *et al.* five-month old rats were used[27]. Rats younger than 20 weeks of age undergo extensive longitudinal growth, which probably affects the outcomes. For osteoporotic research it might be preferable to use skeletally mature animals, so that the results are easier to interpret in relation to the clinical situation.

Use of *in vivo* microCT-scanning allows one to detect very small changes, because longitudinal data are collected. In the control group we observed an absolute decline in BV/TV of 7.5% (SD 1.63) at three weeks (Table 1). At a power of 0.8, an α of 0.05 and with four animals per group, this gives a detectable alternative of 2.5% when comparing means of two groups. Thus, we would have been able to detect an effect of PEMF if the treatment had decreased trabecular bone loss to 5% or less compared to controls. Similar calculations for changes in CtV show that we would have been able to detect an effect of PEMF when cortical thickening was prevented by 0.25 mm^3 or less given that control animals gain 0.6 (SD 0.17) mm^3 of cortical bone in three weeks. In our opinion this is sufficient power because effects smaller than this would most likely not be of clinical significance. With this power we were also able to detect differences when bone changes at the magnitude of other studies were found.

In a clinical trial it was shown that by treating the proximal forearm of osteoporosis-prone women, an increase in BMD was induced at the distal radius of the treated side and also at the contralateral side, which suggests PEMF induced a systemic effect[23]. It is peculiar that this research and the strong beneficial effects in earlier animal experiments have not led to a range of clinical trials in which the effect of this safe and non-invasive intervention on bone mass is further examined. For many years attempts have been made to perform a prospective randomised controlled trial to determine the effect of PEMF on nonunions, but due to a lack of sufficient numbers for adequate statistical power this has never been achieved[17, 22, 37, 38]. Obtaining sufficient numbers would not be a problem in a clinical trial examining the effects of PEMF on bone mass in healthy or osteoporotic individuals. Because PEMF does not induce any side effects and the exact experimental set-up

is important for the outcome, it would be more logical and favourable to perform future studies on the effect of PEMF on bone in humans and not in animals. With the use of non-invasive techniques such as dual-energy X-ray absorptiometry (DXA) scanning and peripheral microCT-scanning the role of PEMF in osteoporosis could be determined with certainty.

In this study we could not demonstrate a positive effect of PEMF on the bone architecture in a ovariectomized rat model of osteoporosis. This is in accordance with earlier published work. However, because there are studies that did find strong effects, it might be that PEMF are very effective under the right circumstances. It might therefore be more practicable to examine the effects of PEMF on bone mass directly in humans such that translation from small animal experiments is not an issue. However, the results obtained in our study do not substantiate a potential role for the use of PEMF in post-menopausal osteoporosis.

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04

Pulsed electromagnetic fields do not affect bone microarchitecture in osteoporotic or healthy rats

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Submitted

ABSTRACT

Pulsed electromagnetic fields (PEMF) are widely used in musculoskeletal disorders. There are indications that PEMF might also be effective in the treatment of osteoporosis. To justify clinical follow-up experiments we examined the effects of PEMF on bone micro-architectural changes in osteoporotic and healthy rats. Moreover, we tested the effects of PEMF on fracture healing. PEMF was examined in rats (age 20 weeks), which were subjected to ovariectomy (OVX) or sham-ovariectomy (sham-OVX) (*n*=16). As putative positive control fibula osteotomies were performed to examine the effects on fracture healing. Treatment was applied to one proximal tibia (3 h/day, 5 days/week), the other tibia was not treated and served as control. Bone architectural changes were evaluated using *in vivo* microCT scans at start of treatment and after 3 and 6 weeks. In both OVX and sham-OVX animals PEMF did not result in cancellous or cortical bone changes during follow-up. PEMF did not affect the amount of mineralized callus volume around the fibula osteotomy. In the current study we were unable to reproduce the strong beneficial findings reported by others. This might indicate that PEMF treatment is very sensitive to the specific set-up, which would be a serious hindrance for clinical use. No support was found that PEMF treatment can influence bone mass for the benefit of osteoporotic patients.

INTRODUCTION

Osteoporosis is a disease characterized by progressive bone loss and deterioration of the microarchitecture leading to an increased fracture risk. Several pharmaceutical treatments are available that aim at reduction of further bone loss or an increase of bone mass. However, these therapies require a regular and probably lifelong intake, with potential side-effects and related high costs. Pulsed electromagnetic fields (PEMF) are also reported to have a beneficial effect on bone microarchitecture and might reduce fracture risk.

PEMF were developed based on the finding that electrical currents exist in mechanically-loaded bone and are important for physiologic regulation of bone metabolism [1]. There is some proof for the effectiveness of PEMF. However, due to the large variation in the characteristics of PEMF signals of different generators, comparison of different *in vitro* and *in vivo* studies is difficult.

Experimental studies examining the effects of PEMF on bone healing in osteotomy gap models have shown the stimulatory effects of PEMF on callus formation and mechanical capacities even when treated for only 1 hour per day[2-5]. The underlying stimulatory mechanisms of electromagnetic fields have been studied *in vitro* using mesenchymal stem cells, osteoblasts or osteoblast-like cells and are related to BMP-2, TGF-beta, IGF-II, prostaglandins, nitric oxide synthase phosphorylation and MAPK activation[6-13].

Although, experimental evidence of the effectiveness of PEMF on bone formation seems quite extensive, clinical evidence is lacking. PEMF are for example widely used for the treatment of nonunions, but their effectiveness is limited to observational studies[14-18] and some smaller control-based studies with sub-optimal study design[19-21]. Therefore the use of PEMF in nonunions is still under debate[22].

Few studies have investigated the potential role of PEMF treatment for osteoporosis. In 1990, Tabrah *et al*. reported a positive effect of PEMF on the bone mineral density (BMD) of radii in older women[23]. Furthermore, few animal studies address the topic. Chang and Chang showed that whole-body PEMF completely prevented OVX induced trabecular bone loss in rats that were treated for 8 hours per day[24]. Cortical bone mass was not affected in this study. Jing *et al*. also demonstrated preventive effects of PEMF on OVX induced trabecular bone loss using BMD analysis[25]. In another study by Sert *et al*. it was shown that electromagnetic treatment induced an almost twofold increase in cortical bone mass in the tibiae of OVX rats[26]. Trabecular bone mass was unfortunately not analyzed.

Since many *in vitro* and *in vivo* studies show strong positive effects of PEMF on bone formation this non-invasive treatment has a potential important role in the treatment of low bone mass and osteoporosis. To justify clinical follow-up experi-

ments we examined the effects of PEMF on the bone microarchitecture using *in vivo* microCT scanning. Using this sensitive technique both cancellous and cortical bone changes can be analyzed in longitudinal and 3D fashion. The effects were examined in ovariectomized and sham-ovariectomized rats. As putative positive control a bilateral fibular osteotomy was added to the model to examine the effect of PEMF on fresh fracture healing.

Methods

16 Female Wistar rats were obtained, age 20 weeks (Harlan, The Netherlands). All animals were housed in the institute's animal facility with a 12-h light/dark regimen, and all received standard food pellets and water ad libitum. The study protocol was approved by the local Animal Experiments Committee (EUR 687) and was in accordance with Dutch law on animal experimentation.

Research model

Three weeks prior to PEMF treatment two groups were made. The animals underwent either a bilateral ovariectomy (OVX) to simulate osteoporosis (*n*=8) or a sham-ovariectomy (*n*=8). This procedure was performed under sterile conditions using gas anesthesia (oxygen with 3% isoflurane; Rhodia Organique Fine Ltd., Bristol, UK). Analgesics were given for three days: 0.05 mg/kg/12 h buprenorphine (Schering-Plough, Kenilworth, NJ, USA). In sham-ovariectomy the procedures were exactly the same, except that the ovaries were only manipulated and not removed.

Nineteen days after (sham-)OVX a bilateral fibula osteotomy was performed in all animals. It has been shown that PEMF stimulated fracture healing using the fibula osteotomy model[5]. To include a positive control, this fresh fracture model was added to our osteoporosis model. In a previous paper we demonstrated that there is no interaction of OVX on mineralized callus volume and vice versa[27]. Under general anesthesia (oxygen/3% isoflurane) the hind legs were shaved. Then, under sterile conditions a 1 cm incision at the lateral side of the calf muscle was made through the skin and fascia. Under microscopic control, the fibula was presented in a blunt manner. At 0.4 cm distal to the fibulo-tibial joint, an osteotomy (including the periosteum) was made using a high-speed mini-saw. The thickness of the osteotomy was the same as that of the saw blade, i.e. 0.1 mm. Fascia and skin were stitched. These animals received analgesics (buprenorphine 0.05 mg/kg/12 h) for two days.

PEMF

PEMF treatment was started 21 days after (sham-)OVX and 2 days after fibula osteotomy. During PEMF treatment the animals were placed in a custom-made harness (Fig. 1). This fixating procedure enabled application of the PEMF coil to the hind leg without the use of an anesthetic. Three weeks prior to the start of PEMF treatment, rats were placed in the harness with an increasing time period to get acclimatized to the harness.

PEMF was given to one hind leg, 5 days/week for 3 h/day. The PEMF signal was produced by a custom-made generator producing a 20 Gauss sinusoid waveform at 50 Hz. The coil extended from the knee to the ankle joint (Fig. 1). The other hind leg was left untreated and served as control. μ-Copper foil was placed around the control leg to exclude any effect of the coil on the contra-lateral side.

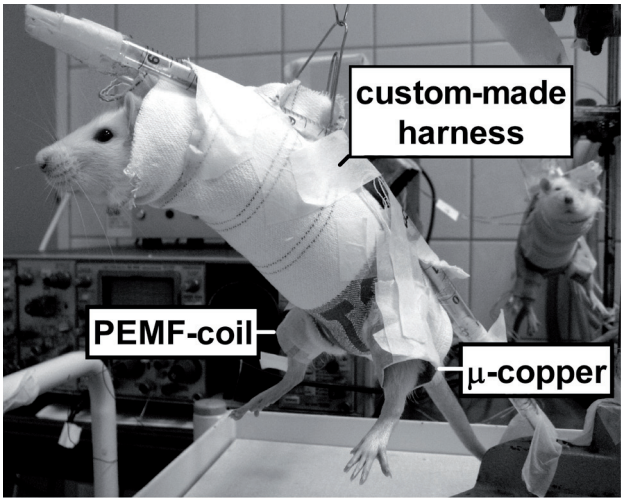

Fig. 1 PEMF treatment. A rat hanging in a custom-made harness receiving PEMF treatment to the right hind leg; the left hind leg is covered with μ-copper foil.

MicroCT scanning

To evaluate bone changes in the proximal tibia, *in vivo* microCT scans were made at start of PEMF treatment and 3 and 6 weeks after PEMF treatment was started, so at 3, 6 and 9 weeks after (sham-)OVX. Under gas anesthesia the hind leg of the rat (from distal femur to the tibial diaphysis) was scanned with an *in vivo* microCT scanner (Skyscan 1076 Microtomograph, Kontich, Belgium) at a voltage of 60 kV, a current of 167 μA and a 0.5 mm aluminum filter, over 196° with a rotation step of 1°, taking 8 min per scan.

Using NRecon (Nrecon software version 1.5, Skyscan) three-dimensional reconstructions (isotropic voxelsize of 18 microns) of two regions of interest were

made, one at the proximal metaphysis which mainly contains cancellous bone and another at the mid-diaphysis, which contains mainly cortical bone (Fig. 2). The reconstruction of the proximal metaphysis was selected manually starting just distally of the epiphysis and continuing until 5.4 mm more distally. The reconstruction of the diaphysis was defined by a region of 3.6 mm starting 9 mm distally from the epiphysis. Bony and non-bony structures were separated using a local threshold algorithm (software freely available)[28] resulting in binary datasets[29]. Cortical and trabecular bone were automatically separated using in-house software. Trabecular architecture in the proximal metaphysis was characterized by determining trabecular volume fraction (BV/TV), connectivity density (Conn/TV), Structure Model Index (SMI) and 3D trabecular thickness (TbTh). Cortical architecture was assessed in the diaphysis and was characterized by cortical volume (CtV) and cortical thickness (CtTh).

Analysis of the mineralized callus formation at the fibula osteotomy was performed on the same microCT scans. In reconstructed datasets the osteotomy site was selected and an area of 2 mm proximal and 2 mm dorsal to the fracture was selected for further analysis (Fig. 2). Because the degree of mineralization within the callus site varied, a global threshold was used. The threshold was set in such way that the cortical bone visually included the selection, in this way the mineralized callus volume was measured.

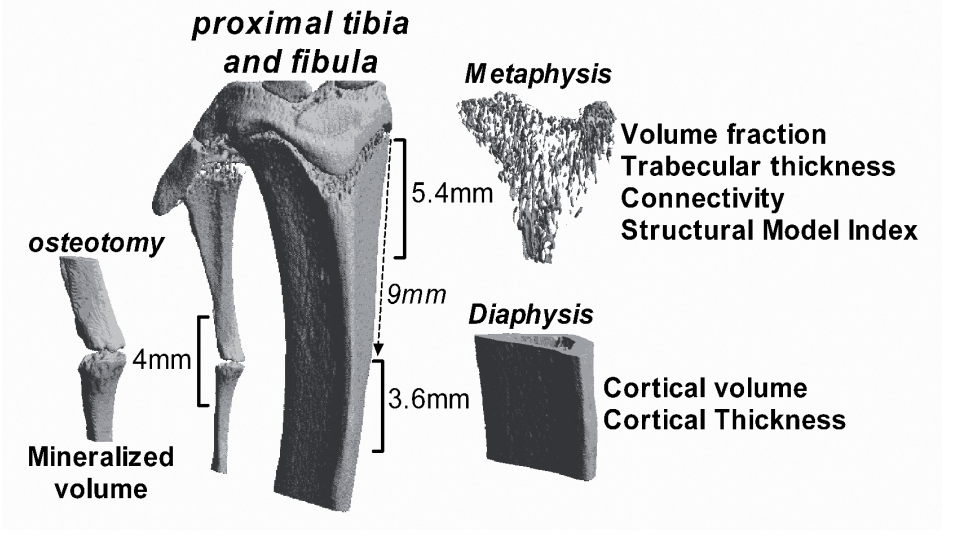

Fig. 2 Micro CT scanning. Three-dimensional reconstruction of a microCT scan of the proximal tibia and the proximal fibula. The analyzed regions of interest are indicated.

Statistics

Differences between the treated legs and the untreated control legs were statistically analyzed for each time point using paired t-tests for all parameters (GraphPad Software, San Diego, CA, USA).

RESULTS

Due to complications during sham-OVX one animal died. In addition, because the fibula osteotomy resulted in additional fractures in the fibula two animals were excluded from analyses of the mineralized callus volume.

At the start of PEMF treatment mean bodyweight in sham-OVX rats was 251.3 g (SD 12.0) and mean increase in bodyweight was 10.7 g (SD 2.9). In OVX animals mean bodyweight was 299.9 g (SD 15.0) at start of PEMF treatment and the mean increase in the 6 weeks follow-up period was 14.1 g (SD 10). None of the animals experienced weight loss during follow-up.

In sham-OVX animals the BV/TV in non-treated control tibias was 27.7% (SD 2.8), 26.9% (SD 3.0) and 25.3% (SD 3.1) at week 0, 3 and 6, respectively; this was not significantly different from PEMF-treated tibias (Fig. 3a). In sham-OVX animals no differences were observed in morphometric or cortical parameters between non-treated control tibias and PEMF-treated tibias at 3 and 6 weeks after start of treatment (Fig. 3b, Table 1).

Mineralized callus formation at the osteotomy site showed a wide variation between and within animals. The average mineralized callus volume declined during follow-up in both the controls and the PEMF-treated tibias but the differences were not significant (Fig. 3c).

In OVX animals the BV/TV in non-treated control tibias was 17.2% (SD 3.2), 12.3% (SD 5.1) and 8.7% (SD 3.0) at week 0, 3 and 6, respectively (Fig. 3a). BV/TV and morphometric parameters showed no significant differences from PEMF-treated tibias at any time point (Table 2). CtV in non-treated control tibias was 25.0 (SD 1.6), 25.7 (SD 1.0) and 26.7 mm^3 (SD 1.8) at 0, 3 and 6 weeks, respectively. Neither CtV nor CtTh were significantly different from that in PEMF-treated tibias (Fig. 3b, Table 2).

Mineralized callus formation showed a wide variation; there were no significant differences between controls and PEMF-treated tibias (Fig. 3c).

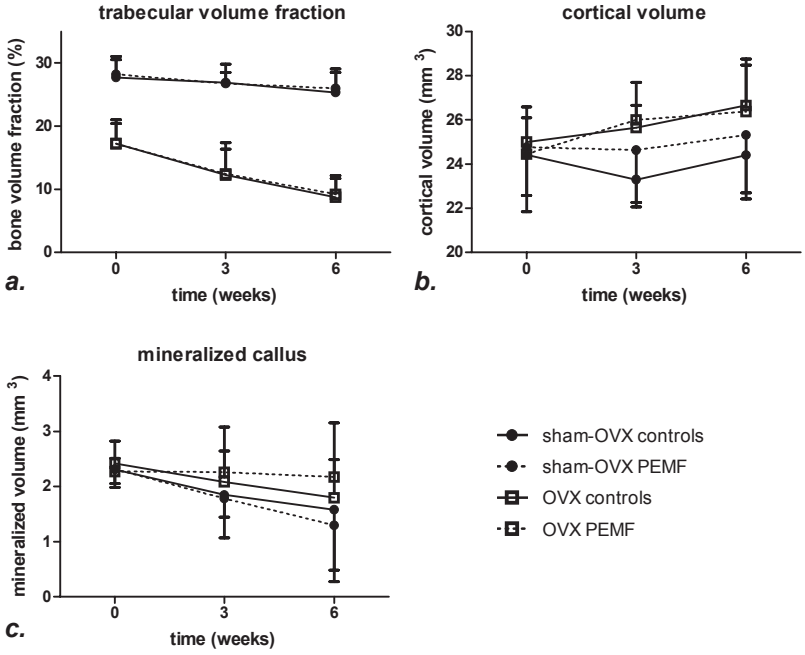

Fig. 3 Bone changes during PEMF treatment. Trabecular volume fraction (a), cortical volume (b) and mineralized callus volume (c) of PEMF-treated and non-treated control tibias in sham-ovariectomized (n=7) and in ovariectomized (n=8) rats. Data are mean values and standard deviations.

Table 1. Bone changes in non-treated and PEMF-treated tibias of sham-ovariectomized rats

	week 3			*week 6*		
	controls	***PEMF***	***p-value***	***controls***	***PEMF***	***p-value***
BV/TV (%)	26.9 (3.0)	26.8 (1.7)	0.42	25.3 (3.1)	25.9 (3.1)	0.68
mean Tr. Thick (*μ*m)	106.7 (5.8)	108 (5.0)	0.19	108.7 (4.1)	109.6 (6.0)	0.74
Conn.dens.(mm³)	75.4 (5.1)	69.5 (10.5)	0.14	63.7 (7.7)	52.0 (10.5)	0.28
SMI	1.85 (0.11)	1.83 (0.18)	0.15	1.87 (0.15)	1.81 (0.24)	0.33
Cort. Vol. (mm³)	23.3 (1.2)	24.6 (2.4)	0.3	24.4 (2.0)	25.3 (2.6)	0.27
Cort. Thick. (*μ*m)	458.5 (11.1)	469.7 (20.4)	0.74	476.6 (18.4)	487.4 (30.0)	0.44

Mean values with standard deviations and p-value (paired t-tests) are given (n=7).

Table 2 Bone changes in the tibia of non-treated tibias and PEMF-treated tibias in ovariectomized rats

	week 3			*week 6*		
	controls	***PEMF***	***p-value***	***controls***	***PEMF***	***p-value***
BV/TV (%)	12.3 (5.1)	12.4 (3.9)	0.78	8.7 (3.0)	9.2 (3.0)	0.85
mean Tr. Thick (*μ*m)	101.0 (6.3)	102.6 (5.74)	0.27	100.3 (3.8)	101.7 (4.8)	0.66
Conn.dens.(mm^3)	16.9 (13.4)	17.1 (10.4)	0.71	8.5 (7.5)	8.7 (7.0)	0.68
SMI	2.50 (0.20)	2.48 (0.18)	0.91	2.62 (0.16)	2.58 (0.17)	0.38
Cort. Vol. (mm^3)	25.7 (1.0)	26.0 (1.7)	0.54	26.7 (1.8)	26.4 (2.4)	0.41
Cort. Thick. (*μ*m)	469.2 (22.0)	477.9 (23.8)	0.45	504.0 (28.4)	495.8 (27.5)	0.24

Mean values with standard deviations and p-value (paired t-tests) are given (n=8).

DISCUSSION

This study examines the effects of PEMF on changes in both cancellous and cortical bone to establish whether PEMF might serve as a treatment for osteoporosis. Although *in vivo* microCT scanning allowed to follow morphometric bone changes in high detail over time, we were unable to detect any differences between PEMF treated and untreated legs.

There are many indications from *in vitro* studies that PEMF might have beneficial effects on the bone architecture at the benefit for osteoporosis. However, few animal experiments using ovariectomized animals exist. Change & Chang showed that OVX induced trabecular bone loss was prevented by PEMF treatment using histomorphometric analyses at 30 days of treatment[24]. PEMF was given with a single pulse at 7.5 Hz and a maximum magnetic field of 8 Gauss for 8 hours per day at the whole animal. In another study using the OVX rat model whole body PEMF lead to an increase of 71% in cortical thickness compared to non-treated controls[26]. PEMF was applied for 4h/day with a 50 Hz sinusoidal waveform of 10 Gauss. Although, the characteristics of PEMF used in these and the current paper is quite comparable, especially between the paper of Sert *et al*. and the current study, we were unable to confirm the pronounced effects of PEMF on cancellous or cortical bone. These contradictory findings suggests that the effects of PEMF on osteoporosis are very sensitive to the specific experimental set-up used. This is confirmed in a more recent paper in which ovariectomized rats that received PEMF during daytime showed a higher BMD than animals receiving PEMF during the night[25]. It is unknown what caused this difference.

With so many pre-clinical experiments on PEMF it is unclear why only one clinical study has examined the effect of this non-invasive and safe treatment on bone mass. Tabrah *et al*. found that the BMD of both the treated and the opposite untreated radius of osteoporotic prone women increased during a 12-week period, suggesting that a systemic effect might be induced by PEMF that influenced BMD[23]. To our knowledge, this study has never been repeated in a randomised controlled trial. According to a review in 2008 more clinical studies are described in Chinese literature[30]. These data suggest that the effects of PEMF on BMD in osteoporosis are controversial.

In contrast to other studies, we examined the effects of locally applied PEMF on osteoporosis. As a putative control we added a fracture model to the osteoporosis model. We demonstrated earlier that there is no interaction between the OVX and the fracture model[27]. Midura *et al*. showed that PEMF stimulated fracture healing at the fibula osteotomy[5]. As in the study of Midura *et al*. we found large variations in mineralized callus volume. However, the amount of nonunions, characterized by progressive gap size due to resorption of the bony ends, was much higher in the current study. In our hands 11 of the 30 fibula osteotomies resulted in a nonunion, irrespective of the study group. We speculate that (micro-)motion is the cause of the large number of failures. Although, the PEMF signal was very well comparable to that of Midura *et al*. (both maximum electrical field of 20 Gauss, treated for 3 h/day), we were unable to confirm the stimulating effects of PEMF on fresh fracture healing.

A limitation of the current study is that only bone microarchitecture was analyzed during follow-up and no mechanical testing or histology was performed. Regarding histological parameters, it is still possible that PEMF affected bone turnover without inducing changes in bone architecture. However, changes in bone turnover which do not translate into improved biomechanical properties are not relevant for osteoporosis since they do not decrease fracture risk.

In conclusion, we could not confirm any positive effects of PEMF for the treatment of osteoporosis or for fracture healing. Although experimental studies have reported strong beneficial effects of PEMF on osteoporosis, these reports were not corroborated by other investigators and did not get a clinical follow-up. If such studies were performed with a negative result, it might not have found its way to literature since researchers have a possible publication bias against negative results[31]. It is also possible that PEMF does work under specific circumstances, but that its effects are highly sensitive to the precise set-up, which would be a serious hindrance to clinical use. However, the present findings provide no evidence to support PEMF as a beneficial treatment for osteoporotic patients.

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Unfocused extracorporeal shock wave therapy as potential treatment for osteoporosis

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ABSTRACT

Extracorporeal shock wave therapy (ESWT) influences the differentiation of bone marrow stroma cells towards osteoprogenitors and increases the expression of several growth factors. To assess whether unfocused ESWT might serve as a treatment for osteoporosis, we examined the bone architecture dynamics of ESWT-treated and untreated rat tibiae using *in vivo* micro-computed tomography (microCT) scanning. In addition, the effects of ESWT on fracture healing, using a bilateral fibula osteotomy, were examined. Unilateral unfocused ESWT with 2,000 pulses and an energy flux density of 0.16 mJ/mm^2 was applied to the hind leg of ovariectomized and sham-ovariectomized rats. A single treatment with unfocused ESWT resulted in a higher trabecular bone volume fraction (BV/TV) in the proximal tibia of the sham-ovariectomized animals. Three weeks after ESWT, BV/TV was 110% of baseline BV/TV in treated legs versus 101% in untreated contralateral control legs (p=0.001) and 105% of baseline BV/TV versus 95% at 7 weeks after ESWT (p=0.0004). In ovariectomized rats, shock wave treatment resulted in a diminished bone loss. At 7 weeks, the BV/TV of the treated legs was 50% of baseline BV/TV, whereas in untreated control legs this was 35% (p=0.0004). ESWT did not influence acute fracture healing. This study shows that bone microarchitecture can be affected by unfocused shock waves, and indicates that unfocused ESWT might be useful for the treatment of osteopenia and osteoporosis.

INTRODUCTION

Osteoporosis is a disease characterized by low bone mass and a deterioration of the microarchitecture of the bone. Consequently, patients suffering from osteoporosis have an increased risk for bone fractures. These fractures are associated with increased morbidity and mortality. Currently treatment is mainly pharmacological. The necessity for lifelong treatment, the potential negative side effects, and the high costs justify the search for alternative treatments. One such treatment might be extracorporeal shock wave therapy (ESWT).

Extracorporeal shock wave therapy is effective in the treatment of nonunions and fresh fractures[1-4]. Shock waves are acoustical pulses that are characterized by a high amplitude (up to 120 MPa), a short rise time (<10 ns), and a (negative) tensile wave (up to 10 MPa)[5]. They can be generated electrohydraulically, electromagnetically, or pneumatically, which has important consequences for the wave characteristics.

In a prospective randomized study, high-energy fractures of the long bones that were treated with ESWT in addition to internal stabilization resulted in a decreased rate of nonunions, less pain, and earlier weight-bearing compared to fractures that only received internal stabilization[4]. Although no prospective doubleblind placebo controlled studies examining the effect of ESWT on delayed and nonunions are available, several observational studies had success rates between 72% and 85%[1-3].

ESWT results in biological responses at an energy flux density (EFD) of 0.16 mJ/mm^2 or higher, and 500 or more pulses[6-8], and might be induced by several mechanisms such as mechanical stimulation, bone marrow hypoxia, subperiosteal hemorrhage, or increased regional blood flow[9-11]. These responses increase the expression of a variety of growth factors, including VEGF-A, IGF-I, TGF-beta, and BMP-2,-3,-4, and -7[6-8]. In studies examining the effects on bone healing, one or more of these growth factors were associated with an increased recruitment of mesenchymal stem cells and an increased differentiation of bone marrow stroma cells towards the osteogenic lineage[6, 7]. Furthermore, ESWT also induces neovascularization[12] and enhances the recruitment of endothelial progenitor cells in ischemic hind limbs[13].

ESWT is noninvasive and is used in a wide variety of musculoskeletal disorders. The development of non-focusing shock wave generators that act at a relatively large region further expanded its use to dermatologic conditions, such as diabetic ulcers, and acute and chronic wounds[14]. An accompanying advantage of these non-focused shock waves is that they are less painful for the patient, so analgesia is not required, making this application easily accessible to the clinical practice.

Also, this further implies that, with unfocused ESWT, larger areas of bone can be treated, enabling opportunities for the treatment of osteoporosis.

In this study, we have examined the effects of non-focused electrohydraulically generated shock waves on the bone architecture dynamics of ovariectomized and control rats to determine if non-focused ESWT can serve as a treatment for osteoporosis. Additionally, the effects of ESWT on fracture healing, using a bilateral fibula osteotomy, were examined.

MATERIALS AND METHODS

Animal Models and Surgical Procedures

Thirty-six 13-week-old female Wistar rats (Harlan Netherlands BV, Horst, the Netherlands) were housed in the animal facility of the ErasmusMC, with a 12-h light–dark regimen, at 21°C during the experimental period. Animals received standard food pellets and water ad libitum. All procedures were examined and approved by the animal experiments committee of the institution (EUR 939) and conformed with Dutch law on animal experiments.

Six groups were examined; all groups consisted of six rats. At 20 weeks of age, the animals received an ovariectomy (OVX) to simulate osteoporosis or a sham-ovariectomy (sham-OVX), in which the operative procedure was the same except that the ovaries were left intact. Since positive effects of ESWT on fresh fracture healing have been described[4, 15-18], we added a fracture model as a positive control in the study and analyzed the effects of unfocused ESWT on fracture healing as well.

Three groups received ESWT at the right tibia (Fig. 1). In addition to the sham-OVX rats, all rats that received ESWT also received a bilateral fibular osteotomy to examine the effects of unfocused ESWT on fracture healing. Group A consisted of sham-OVX rats (non-osteoporotic) that received 2,000 shock waves 3 weeks after sham operation. Group B consisted of OVX rats that received 2,000 shock waves 3 weeks after OVX. Group C consisted of OVX rats that received 1,000 pulses 3 weeks after OVX and received another 1,000 pulses 3 weeks later. This group was added to examine whether two repetitive ESWT treatments would affect bone mass more than a single treatment.

weeks / group	-3	0	3	7
		µCT	µCT	µCT
A	Sham-OVX	**#** + ESWT 2000 pulses *		†
B	OVX	**#** + ESWT 2000 pulses *		†
C	OVX	**#** + ESWT 1000 pulses *	ESWT 1000 pulses	†
X	OVX	**#**		†
Y	OVX			†
Z		**#**		†

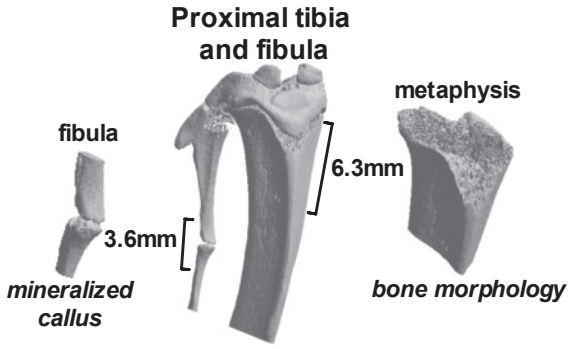

Fig 1 Schema of the study design representing the treatment groups and the analyzed regions (*, unilateral to the right leg; #, a bilateral fibula osteotomy; {†, sacrifice).

To evaluate a potential effect of the fibular osteotomy on bone loss and vice versa, another three control groups were added. The first got both a bilateral OVX and fibular osteotomy, the second got only an OVX, and the third group got only a bilateral fibular osteotomy.

The osteotomy was performed 2 days prior to the first shock wave treatment. Because the fibula is proximally attached to the tibia with a syndesmosis, and at the distal side with a bony union, a stabilized fracture can be created[19, 20]. A 1-cm incision at the lateral side of the calf muscle was made through the skin and fascia. Under microscopic view, an osteotomy, including the periosteum, was made using a high-speed mini saw 0.4 cm distal to the fibulotibial joint. The osteotomy thickness was the same as that of the saw blade, 0.1 mm. Fascia and skin were stitched. All operative procedures were performed under sterile conditions with general anesthesia (O_2 with 2% isoflurane; Rhodia Organique Fine Ltd., Bristol,

UK). Analgesics were given for 3 days as in 0.05 mg/kg/12 h buprenorfine (Schering Plough, Kenilworth, NJ).

Extracorporeal Shock Wave Therapy

Unfocused, electrohydraulically generated shock waves with a treatment area of 3.8 cm in diameter, an energy flux density of 0.16 mJ/mm^2, and a frequency of 5 Hz were given using a commercially available generator (Dermagold/Orthowave 180, Tissue Regeneration Technologies, Woodstock, GA). After general anesthesia was established, both hind legs were shaved from ankle to knee. The rat was placed on its left dorsal-lateral side when the right tibia was treated. The applicator was placed at the anterolateral side of the hind leg, covering the surface from the proximal to the distal tibia. An ultrasonic gel was used as coupling media between the applicator and the skin. The contralateral left tibia served as a control and was not treated. Animals did not receive additional analgesics during or after treatment.

Analyses of Morphologic Parameters and Mineralized Callus Volume

In vivo micro-computed tomography (microCT) scanning was performed under gas anesthesia (isoflurane/oxygen)[21]. In supine position, the hind leg of the rat was fixed, allowing scanning of both the proximal tibia and the osteotomy site in a single session. Scanning was performed with a resolution of 18 mm using a Skyscan 1076 microtomograph (Kontich, Belgium) at a voltage of 60 kV, a current of 167 µA, and a 0.5-mm aluminium filter, over 196° with a 1° rotation step, taking 8 min. per scan.

For assessment of changes in cancellous and cortical bone, 3D reconstructions of the proximal tibia were made (Nrecon software version 1.5, Skyscan). To discriminate bony structures from non-bony structures, binary datasets were made using a local threshold algorithm (3D Calculator software)[22, 23].

Analyses were made on the proximal 6.3 mm of the tibial metaphysis, which was manually selected. Cortical and trabecular bone were automatically separated using in-house software. Trabecular architecture was characterized by determining trabecular volume fraction (BV/TV), connectivity density, Structure Model Index (SMI), and 3D Trabecular Thickness. Cortical architecture was characterized by Cortical Volume and Cortical Thickness.

For assessing mineralized callus volume, 3D reconstrutions of the fibula were made using the same software. The osteotomy site and areas of 1.8 mm proximal and dorsal were selected for further analysis. A global threshold at the cut-off point of cortical bone was used to select mineralized callus. The mineralized volume was measured using 3D Calculator software.

Histology

Directly after euthanasia, the hind legs were harvested and fixed in paraformaldehyde. The proximal half of the tibia was dehydrated and block embedded in methylmethacrylate; 6-mm thick sagittal sections were made throughout the proximal tibia. Overall appearance and new bone formation was evaluated using a thionine staining (0.05% thionine in 0.01 M aqueous sodium phosphate, pH5.8 for 5 min). Sections that were stained in 2% Toluidine blue were analyzed under polarized light to observe the presence of woven bone.

Statistics

Results are presented relative to baseline at start of treatment (time point, t=0, is 100%). In the treatment groups, differences between means of the ESWT-treated right side and the untreated left side were evaluated for all parameters using paired t-tests (GraphPad Software, San Diego, CA). In the control groups that did not receive ESWT, differences between group means of all parameters were evaluated using the Mann–Whitney U test.

RESULTS

The surgical procedures did not result in complications. Directly after ESWT, redness of the skin and minor bleedings were observed at the treated site. When rats awoke from anesthesia, they did not use the treated leg directly. This rapidly improved, and after 10 min no difference was observed between treated and untreated legs. No weight loss occurred the days following ESWT. The average weight gain over the 10 weeks of the experimental was 85.2 g (67–121 g) in treated OVX rats and 77.8 g (63–89 g) in control OVX rats, which was not significantly different (p=0.36). In treated sham-OVX rats, the weight gain was 21.8 g (4–31 g), and in control sham-OVX rats, 23.8 g (15–34 g) (p=0.34).

No difference in cancellous and cortical bone morphometric parameters was found between OVX rats with a bilateral fibular osteotomy and rats that received ovariectomy only (Table 1). Bone healing at the osteotomy site was not significantly different in OVX or sham-OVX rats (Table 2).

Table 1 Trabecular bone volume fraction

	BV/TV in ovariectomy group with osteotomy (n = 6)	BV/TV in ovariectomy group with sham-osteotomy (n = 6)	p-value
week 0	0.19 (0.06)	0.17 (0.03)	0.54
week 3	0.13 (0.03)	0.12 (0.02)	0.70
week 7	0.10 (0.03)	0.07 (0.02)	0.18

Average Trabecular Bone Volume Fraction (BV/TV) in control groups (± SD)

Table 2 Mineralized Callus Volume

	Callus Volume in ovariectomy group with osteotomy (n = 6)	Callus Volume in ovariectomy group with sham-osteotomy (n = 6)	p-value
week 0	2.2 (0.36)	2.2 (0.26)	0.94
week 3	2.0 (0.8)	1.9 (0.59)	0.82
week 7	1.7 (1.6)	1.6 (1.1)	1.0

Average Mineralized Callus Volume (mm^3) in control groups (± SD)

Microarchitectural Bone Changes after ESWT

In sham-OVX rats (group A), BV/TV was 0.26 (range 0.21-0.30) at start of ESWT treatment. After 3 weeks of treatment, BV/TV of the ESWT-treated legs was 110% of baseline, and BV/TV of untreated control legs was 101% of baseline (Fig. 2a), a significant increase in difference (p=0.001). Seven weeks after treatment, BV/TV was 105% of baseline in treated legs, whereas the non-ESWT treated control side lost bone to 95% of baseline, a significant decrease in difference (p=0.0004).

At baseline, connectivity density in the legs of sham-OVX rats was 66.5 (range 49.4-78.6). At 3 and 7 weeks after treatment, it increased in the treated legs compared to the untreated control legs (p=0.03 and p=0.018, respectively) (Fig. 3). The SMI (1.8 on average at baseline), in which an index of 3 indicates the presence of rods and an index of 0 indicates the presence of plates, was significantly lower in the treated legs compared to untreated control legs at 3 weeks (p=0.008), indicating that the structures were more plate-like in the treated legs (Fig. 3). Cortical volume and trabecular thickness were not different in the treated legs compared to control legs.

In OVX rats that received 2,000 shock waves at one time point (group B), BV/TV was 0.19 (0.16–0.24) at start of treatment. Three weeks after treatment, 83% of the BV/TV at baseline remained in the treated legs (Fig. 2b). BV/TV in the non-ESWT control legs was 74% of baseline. The difference between treated and control legs was not significant (p=0.12). Seven weeks after treatment, the BV/TV was 50% in the treated legs and 35% in the control legs, a significant difference (p=0.0004). At 7 weeks, bone loss of the treated leg was diminished compared to the non-treated

control leg in every animal. The morphometric parameters and the cortical bone were not affected by ESWT.

In OVX rats that received 2 times 1,000 shock waves (group C), BV/TV was 0.17 (0.12-0.22) at start of treatment. Three weeks after treatment, BV/TV was 84% of baseline (Fig. 2a). In the control legs, BV/TV was 80%, which was not significantly different (p=0.26). Seven weeks after treatment, BV/TV in the treated legs was 54% (43.2%–67.9%) of baseline, whereas BV/TV in the control legs was 44%, not a significant difference (p=0.13). Again, the morphometric measurements and cortical bone were not affected by ESWT.

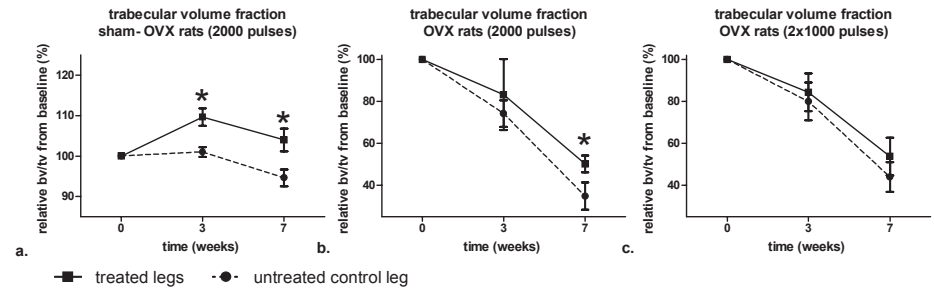

Fig. 2 BV/TV as percentage from baseline in sham-OVX rats receiving 2,000 pulses (a), and OVX rats (b, c) receiving 2,000 (b) or 2 x 1,000 (c) pulses. (*, p<0.05.)

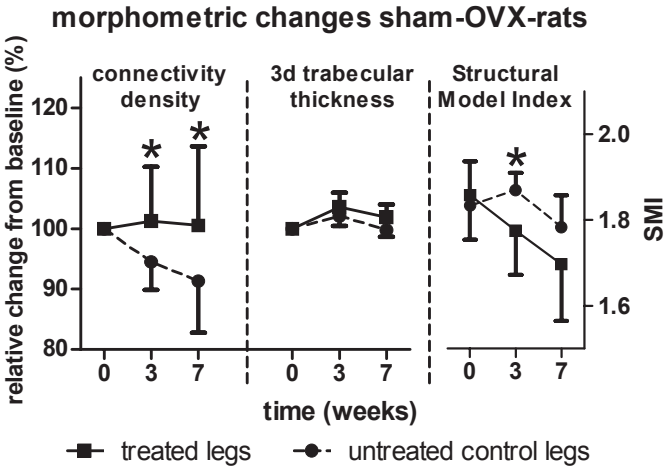

Fig. 3 Morphometric bone changes in sham-OVX rats. Connectivity density and 3D trabecular thickness as percentage of baseline. For SMI, 3 indicates the presence of rods and 0 indicates the presence of plates.

Mineralized Callus Formation

In all treatment groups, a wide variation was seen in the amount of mineralized callus both in the ESWT-treated and the untreated control legs (Fig. 4). At 3 and 7 weeks after ESWT, no beneficial or unfavorable effect of ESWT on bone healing in sham-OVX (p=0.10 and p=0.16, respectively) or OVX rats (group B, p=0.20 and p=0.08, respectively; group C, p=0.82 and p=0.79, respectively) could be found.

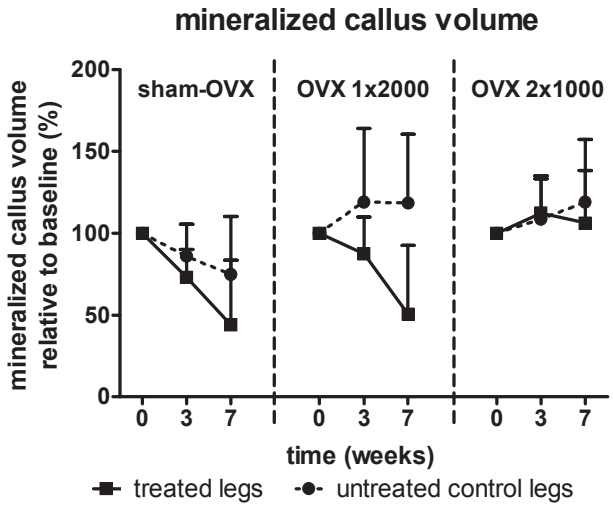

Fig. 4 Mineralized callus volume presented as percentage from baseline in sham-OVX rats receiving 2,000 shock waves (sham-OVX), and OVX rats receiving 2,000 shock waves (OVX 1 x 2,000) or two times 1,000 shock waves (OVX 2 x 1,000).

Histology

No differences in mineralization or osteoid appearance was observed between untreated control and treated legs in thionine-stained sections of the proximal metaphysis. Examination of toluidine-stained sections under polarized light did not show the presence of woven bone in any of the shock wave treated legs (Fig. 5).

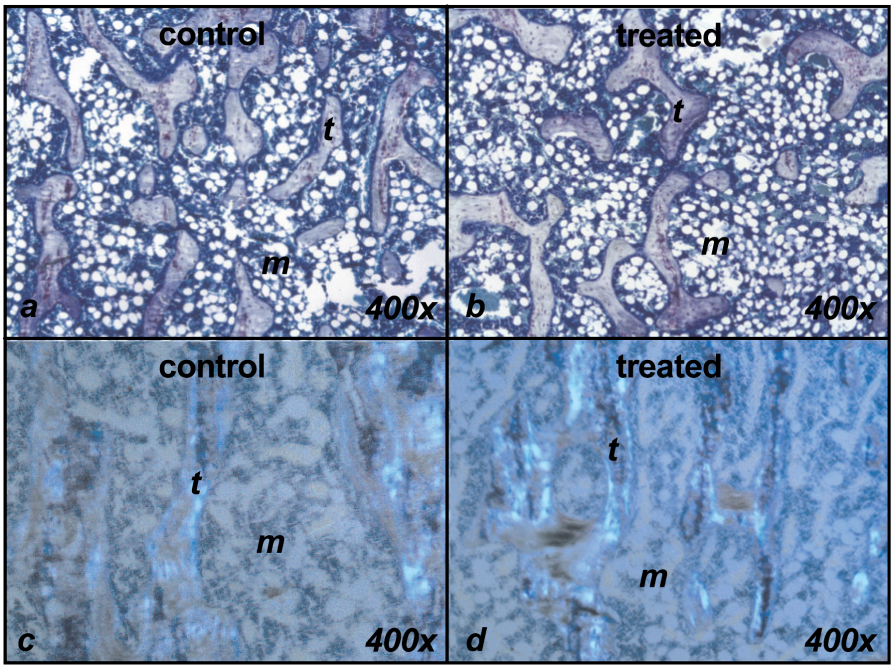

Fig. 5 Sections of the proximal metaphysis of control (a,c) and shock wave treated leg (b,d). Thionine stained sections (a,b) and toluidine blue stained sections under polarized light (c,d). No woven bone could be detected when shock wave treated or untreated bones were carefully examined under polarized light.

DISCUSSION

We examined the effects of unfocused shock waves on the microarchitecture in an osteoporosis rat model and sham control. A single treatment with unfocused electrohydraulically generated shock waves resulted in increased trabecular bone volume and diminished age-dependent bone loss in healthy, non-osteoporotic bone. Moreover, unfocused shock waves with 2,000 pulses in one session diminished trabecular bone loss in ovariectomized rats with established bone loss treatment at 3 weeks post-OVX. These data suggest that ESWT may be a potential treatment for osteopenia and osteoporosis.

Several studies showed the effectiveness of shock wave therapy in healing of fresh fractures[4, 15-18]. Because this was the first time shock wave therapy was performed in an osteoporosis model, a positive control was added, allowing to conclude the potential ineffectiveness on osteoporosis in the presence of an osteogenic stimulator. Therefore, we added a fresh fracture model as a positive control. Although the usefulness of this model was described in other studies examining

biophysical stimuli[19, 20], we found a wide variation with a high number of nonunions within all groups, including the untreated control legs and control groups. This variation might have contributed to the negative finding of ESWT on fresh fracture healing. Retrospectively, this model did not serve as a positive control and might, unfortunately, hamper the interpretation of our results. However, mineralized callus was observed in many animals, concluding that, at least in this model, unfocused shock waves were ineffective. Besides, all previous studies used focused shock waves instead of unfocused shock waves, so the lack of an effect might also be explained by this difference. Furthermore, as shown in our control groups, the bilateral fibular osteotomy did not affect BV/TV, therefore BV/TV findings can be interpreted irrespectively of the fracture model.

We did not analyze the mechanism behind the biological effects. Minor bleeding and a transient disuse of the treated leg were seen directly after ESWT therapy, as described in other studies[9, 24]. Disruption of trabeculae or fracture of the cortex were described when focused shock wave therapy with high energy levels (0.54–0.9 mJ/mm^2) or when a high number of pulses (1,500) were applied[9, 10, 25]. However, these side effects occur in a dose-related manner, and since we used unfocused shock waves with an energy flux density of 0.16 mJ/mm^2, it is not surprising that we did not find these side effects[10, 24]. Our results contribute to the assumption that shock waves induce biological responses without gross damaging effects[6-9, 11].

Although bone loss was diminished by ESWT, the effects were small, of the magnitude of a low-dose bisphosphonate treatment[26]. In sham-OVX rats, only connectivity density and SMI were affected, and in OVX rats, no morphometric parameters were significantly different in shock wave-treated legs. A limitation of our study is that we only examined shock waves with an EFD of 0.16 mJ/mm^2. We assume that optimizing the treatment protocols in terms of pulse number and EFD might increase the effectiveness of this therapy. We examined if the effect of a single treatment was different from the effect of two treatments, keeping the amount of shock waves the same in the two conditions. We could find a significant effect in using 2,000 pulses in one treatment, but no significant effect when two treatments of 1,000 pulses were applied. This suggests that the number of shock waves applied in one session is more important for the therapeutic effect. Whether a higher number or higher energy flux densities are more effective should be examined. Finally, it would be interesting to examine whether shock waves are effective in combination with other pharmaceutical osteoporotic treatments.

We demonstrate that unfocused shock wave therapy can induce bone formation in healthy bone, whereas in OVX, estrogen-deficient rats with established bone loss (3 weeks after OVX), bone loss can be diminished. We used an osteoporosis model with established bone loss because it might be a better representation of the

clinical situation than when treatment is given directly after estrogen deficiency is created. In additional experiments in which animals were treated with unfocused ESWT 10 weeks after OVX (2,000 shock waves and an energy flux density of 0.16 mJ/mm^2), in which the trabecular bone volume fraction was 8% (6%-11%) at time of treatment, no effect was found (data not shown). This might lead to the idea that the more pronounced effects in the sham-OVX animals than in the animals with established bone loss might be related to the amount of bone that remained rather than the estrogen status of the animals. This suggests that unfocused shock waves mainly affect the bone dynamics of existing surfaces and does not induce *de novo* bone formation. In patients with osteoporosis, a distinctive amount of trabeculae are left in the hip and vertebrae. Therefore, ESWT might be effective both in osteopenia and osteoporosis[27-29].

A limitation of our study is the difficulty in comparing the effect of the sham-OVX and OVX rats, since the amount of bone tissue volume was much lower at the time of treatment in the OVX animals, and therefore the energy of shock waves was distributed differently, which might lead to other biological responses.

Our current findings indicate that unfocused shock waves can play a role in osteopenia and osteoporosis and justify further experiments on the effects of unfocused ESWT on bone. Clinically, unfocused shock waves can be applied without anesthesia to skeletal sites that are specifically prone for fracturing, which might contribute to a reduction in fracture risk.

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06

Unfocused extracorporeal shock waves induce anabolic effects in rat bone

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ABSTRACT

Extracorporeal shock waves are known to stimulate the differentiation of mesenchymal stem cells toward osteoprogenitors and induce the expression of osteogenic-related growth hormones. The aim of this study was to investigate if and how extracorporeal shock waves affected new bone formation, bone microarchitecture, and the mechanical properties of bone in a healthy rat model, in order to evaluate whether extracorporeal shock wave therapy might be a potential treatment for osteoporosis. Thirteen rats received 1000 electrohydraulically generated unfocused extracorporeal shock waves to the right tibia. The contralateral, left tibia was not treated and served as a control. At two, seven, twenty-one, and forty-nine days after administration of the shock waves, *in vivo* single-photon-emission computed tomography (SPECT) scanning was performed to measure new bone formation on the basis of uptake of technetium-labeled methylene diphosphonate (^{99m}Tc-MDP) (n=6). Prior to and forty-nine days after the extracorporeal shock wave therapy, micro-computed tomography (microCT) scans were made to examine the architectural bone changes. In addition, mechanical testing, microcrack, and histological analyses were performed. Extracorporeal shock waves induced a strong increase in ^{99m}Tc-MDP uptake in the treated tibia compared with the uptake in the untreated, control tibia. MicroCT analysis showed that extracorporeal shock waves stimulated increases in both trabecular and cortical volume, which resulted in higher bone stiffness compared with that of the control tibiae. Histological analysis showed intramedullary soft-tissue damage and *de novo* bone with active osteoblasts and osteoid in the bone marrow of the legs treated with extracorporeal shock waves. Microcrack analysis showed no differences between the treated and control legs. This study shows that a single treatment with extracorporeal shock waves induces anabolic effects in both cancellous and cortical bone, leading to improved biomechanical properties. Furthermore, treatment with extracorporeal shock waves results in transient damage to the bone marrow, which might be related to the anabolic effects. After further examination and optimization, unfocused extracorporeal shock waves might enable local treatment of skeletal sites susceptible to fracture.

INTRODUCTION

Current pharmaceutical treatments for osteoporosis interfere in the remodeling cycle by blocking osteoclastic resorption or changing the balance between bone resorption and formation. These therapies require regular intake of medication and are accompanied by potential adverse side effects. Biophysical stimuli such as mechanical vibration, ultrasound, and pulsed electromagnetic fields have been suggested as an alternative treatment, but these stimuli seem to have too little effect on bone remodeling and bone architecture[1-4]. Another potentially useful biophysical intervention that has proven effective for acute fractures[5] and nonunions[6-8] is treatment with extracorporeal shock waves.

Shock waves are acoustical pulses that are characterized by a high amplitude (>120 MPa) and a short rise time (<10 ns) and are followed by a longer low-magnitude negative wave (>10 MPa)[9]. Focused shock waves have been reported to alter bone turnover or induce trabecular or cortical bone formation in animal models[10-12]. Several mechanisms for triggering these anabolic effects have been suggested. First, the increased bone formation is associated with an increased expression of several growth factors (including bone morphogenetic proteins (BMPs), transforming growth factor-beta (TGF-b), and vascular endothelial growth factors (VEGF) and the activation of cell signaling pathways like ERK and Wnt[13-17]. Second, shock waves stimulate both the recruitment and the differentiation of mesenchymal stem cells[13, 16, 18]. Finally, it is suggested that the effects are triggered by damage, since periosteal detachment, cortical fractures, and disruptions of trabeculae are observed after application of extracorporeal shock wave treatment[10, 19, 20].

In all of the experimental studies cited above, the investigators applied focused shock waves to a small area. However, since the skeletal sites that need to be treated in osteoporosis are much larger than the area encompassed by focused shock wave therapy, it is difficult to apply focused shock wave therapy for the treatment of osteoporosis. Transducers that apply unfocused shock waves are now available. These transducers have a treatment zone of 3.8 cm in diameter, allowing treatment of skeletal sites that are prone to fracture in patients with osteopenic conditions. However, it is unknown whether, and for how long, unfocused shock waves can influence bone remodeling, bone microarchitecture, and the mechanical properties of bone.

While the overall research aim is to explore the possibilities of using extracorporeal shock waves for osteoporosis therapy, the purpose of this particular study was to investigate whether, and how, unfocused extracorporeal shock wave therapy affects new bone formation, bone microarchitecture, and the mechanical properties of bone in a healthy rat model.

MATERIALS AND METHODS

Animals and Treatment Protocol

Six male Wistar rats (fifteen weeks of age) obtained from Harlan Laboratories (Horst, The Netherlands) underwent extracorporeal shock wave therapy and were followed for forty nine days with single-photon-emission computed tomography (SPECT) scanning and *in vivo* micro-computed tomography (microCT) scanning. Seven additional rats were used in other experiments to examine osseous and soft-tissue microdamage. Those seven animals were killed twenty-four hours after administration of the extracorporeal shock wave therapy. The six rats used for ongoing study were housed sometimes in pairs and sometimes in threesomes at the animal facility of the Erasmus MC, with a twelve-hour light-dark regimen, at 21°C. The animals received standard food pellets and water ad libitum. The research protocol (number 116-07-02) was approved by the local committee for animal experiments and is in accordance with Dutch law.

The sample size was chosen for logistical reasons and not on the basis of a priori hypothesized effect-size and variance estimates. Thus, the analyses are exploratory, and effects that were not found to be significant should not be presumed to be clinically unimportant. These data provide effect and variance estimates for future work.

Extracorporeal Shock Wave Therapy

Both hind legs of the anesthetized rat were shaved, and the rat was placed on its left dorsolateral side. An ultrasonic gel that served as coupling medium was applied to the right hind leg. The lithotripter of the shock wave device (Dermagold; Tissue Regeneration Technologies, Woodstock, Georgia, manufactured by MTS, Konstanz, Germany) was placed at the anterolateral side of the right hind leg and covered the whole tibia. A total of 1000 electrohydraulically generated extracorporeal shock waves were applied at 3 Hz with an energy flux density of 0.3 mJ/mm^2. The contralateral, left tibia served as a control and was not treated.

Multi-Pinhole SPECT Scanning

At two, seven, twenty-one, and forty-nine days after the extracorporeal shock waves were applied, the local bone formation was analyzed with use of multi-pinhole (mph) SPECT[21]. At four hours prior to scanning, 185-MBq (4995-μCi) technetiumlabeled methylene diphosphonate (^{99m}Tc-MDP) was intravenously injected. ^{99m}Tc-MDP first binds to the unmineralized extracellular matrix of newly formed bone and is subsequently irreversibly incorporated into the mineralized extracellular matrix. The animals were scanned with use of a dedicated small-animal mph-SPECT scan-

ner (Nanospect; Bioscan, Washington, DC). During scanning, the animals were anesthetized with isoflurane (2%). To minimize interference with the radioactive signal from the body, the hind legs were stretched and were fixed in a custom-made wooden frame so that both tibiae could be scanned at the same time.

The total scanning time was twenty-six minutes. Four gamma cameras, each containing nine pinholes of 2.5 mm in diameter, were used. Acquired scan data were reconstructed at a voxel size of 0.33 mm. The images were rotated so that all tibiae appeared with the same orientation. Two regions of interest were determined in the reconstructed image: the first (5 mm in width) was positioned 1 mm distal to the active signal of the growth plate, and the second (7 mm in width) was positioned at the diaphysis 8 mm distal to the growth plate (Fig. 1). The amount of radioactive tracer uptake was quantified within each region of interest.

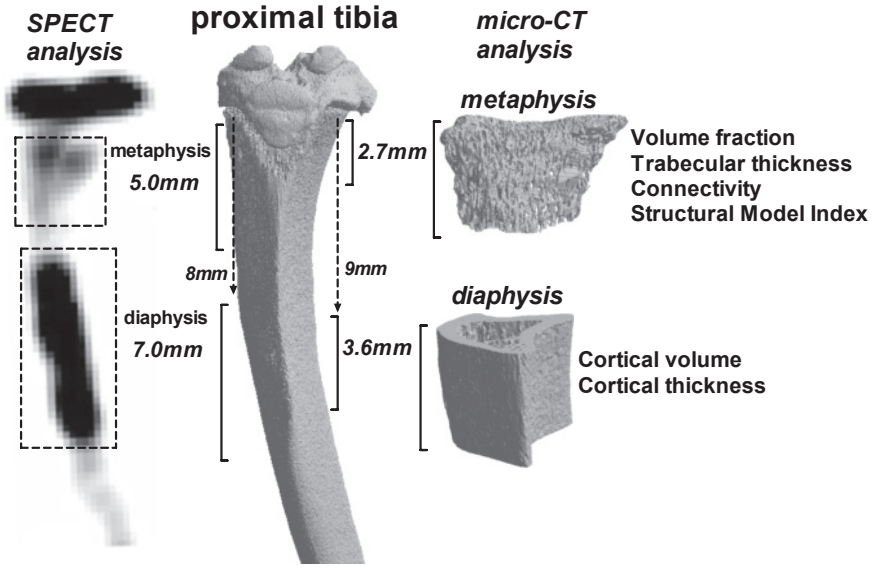

Fig. 1 Schematic representation of the regions of interest that were analyzed for the SPECT scan analysis and the microCT analysis.

In Vivo MicroCT Scanning

Prior to and forty-nine days after extracorporeal shock wave therapy, *in vivo* microCT scans were made with the animal under anesthesia. The hind leg of the rat was fixed in a supine position, allowing a 17-mm scan of the proximal part of the tibia. Scanning with a Skyscan 1076 Scanner (Kontich, Belgium) was performed at a voltage of 60 kV, at a current of 167 μA, and with use of a 0.5 mm aluminium

filter, over 196° with a rotation step of 1° , with each scan taking eight minutes. This resulted in data sets with an isotropic voxel size of 18 mm. NRecon software (version 1.5, Skyscan) was used to make three-dimensional reconstructions of two regions of interest, similar to the regions of interest for the mph-SPECT scans. The reconstruction of the proximal metaphysis included the region starting just distal to the physis and continued distally for 2.7 mm (Fig. 1). The reconstruction of the diaphysis was defined by a region of 3.6 mm, starting 9 mm distal to the physis. Osseous and nonosseous structures were separated with use of a local threshold algorithm (software freely available)[22] resulting in binary data sets[23]. Cortical and trabecular bone were automatically separated with use of in-house software. Trabecular architecture in the proximal metaphysis was characterized by determining the trabecular volume fraction (trabecular bone volume divided by total volume), connectivity density (connectivity divided by total volume), structure model index (in which an index of 3 indicates the presence of rods and an index of 0 indicates the presence of plates), and three-dimensional trabecular thickness. Cortical architecture was assessed in the diaphysis and was characterized by cortical volume, three-dimensional cortical thickness, and the periosteal and endosteal perimeters.

Three-Point Bending Tests

Mechanical properties were determined with use of three-point bending. At day 49, the animals were killed with use of an overdose of pentobarbital. The tibiae were dissected, the soft tissue was removed, and the specimens were stored in phosphatebuffered saline solution at 4°C. Testing with a Single Column Lloyd LRX Testing System (Lloyd Instruments, Hampshire, United Kingdom) was performed with the bones equilibrated to room temperature. Each tibia was stably positioned on its medial anterolateral site. The distance between the loading posts was 2.36 cm; in this way, one post was just distal to the condyles and the other was just proximal to the malleoli. To ensure a stable position of the bone, five preconditioning runs with a displacement rate of 0.01 mm/s and a maximal force of 4 N were made, after which the load returned to the position corresponding to 0 N[24]. Thereafter, the fracture test was carried out with a displacement rate of 0.01 mm/s and a maximal displacement of 3.0 mm. Displacement (mm) and force (N) were registered at a sample rate of 20 Hz. Force displacement graphs were made, and load to failure, toughness, stiffness, and the Young modulus were determined. The Young modulus was determined with the equation E = F/D*l3/Ix , in which F/D is the stiffness, l is the length between the loading posts, and Ix is the moment of inertia around the bending axis. The moment of inertia was determined from the microCT cross section that corresponded with the fracture site. This cross section was rotated so that the moment of inertia around the bending axis (Ix) could be calculated with CT analyzer software (Skyscan).

Histological Analysis

Directly after mechanical testing the hind legs were fixed in 4% paraformaldehyde. After twenty-one days, the proximal halves of the tibiae were dehydrated and were block-embedded in methylmethacrylate. Sagittal sections (6 µm thick) were made of the entire proximal part of the tibia. The overall appearance was evaluated with hematoxylin and eosin (H & E) staining (Gill's hematoxylin for five minutes and eosin for one minute), and new bone formation was evaluated with use of thionine staining (0.05% thionine in 0.01 M aqueous sodium phosphate, pH 5.8, for five minutes). To evaluate the bone marrow specifically, May-Grünwald-Giemsa staining was performed (May-Grünwald stain for five minutes and Giemsa stain for twenty minutes). The number of adipocytes was counted in an area of 2.25 cm^2 in three different matched sections of treated and control samples. Accordingly, the diameter of fifty adipocytes was measured.

Perls' staining to detect the presence of hemosiderin (an indication that bleeding has occurred) was performed by incubating the sections in 2% hydrochloric acid and 2% potassium hexacyanoferrate (Klinipath, Duiven, The Netherlands) for twenty minutes. After they were rinsed in distilled water, the samples were counterstained with pararosaniline (0.02%).

To analyze the presence of periosteal detachment, subperiosteal hemorrhage, and intramedullary damage, three additional rats were administered extracorporeal shock wave therapy as described above. At twenty-four hours after the extracorporeal shock wave therapy, both the treated and the untreated hind legs were harvested and processed as described above. H & E and Perls' staining was then performed.

Microcrack Analysis

To assess whether extracorporeal shock wave therapy had induced microdamage to the bone, four additional animals were obtained. Again, extracorporeal shock wave therapy was applied to one leg, and the contralateral (untreated) leg served as the control. At twenty-four hours after extracorporeal shock wave therapy, the animals were killed and the tibiae were dissected and fixed in formaldehyde (4%).

The tibiae were stained with en bloc basic fuchsin staining and embedded in methylmethacrylate[25]. The proximal part of the tibia was cut in 150-mm-thick frontal sections with use of a diamond wire saw (Histo Saw; Delaware Diamond Knives, Wilmington, Delaware). Bone measurement was performed at 200x· magnification of an 8 to 10-mm^2 region of secondary spongiosa 1 mm away from the growth plate with use of the Bioquant digitizing system (R&M Biometrics, Nashville, Tennessee). Stained microcracks were defined by sharp edges, some depth of field, and permeation of stain into the crack walls. Measurements included crack density

(crack number/mm^2 of bone area), mean crack length (in mm), and crack surface density (crack number x crack length/bone area, in mm/mm^2).

STATISTICAL METHODS

Statistical analysis was performed with GraphPad Prism Software (GraphPad Software, San Diego, California), with use of paired testing, in which the treated and untreated (control) legs of each animal served as a pair. The results of the mph-SPECT scans were statistically analyzed with use of the Wilcoxon matched-pairs test, since the uptake of radionuclide was not normally distributed. All other analyzed parameters were normally distributed, and statistical analysis was performed with use of paired t tests. In the analysis of the results of the microCT scanning, differences between the findings at the start and the end of the experiment were also assessed with use of paired t tests, in which two time points (day 0 [prior to the extracorporeal shock wave therapy] and day 49) of one leg served as a pair.

RESULTS

Extracorporeal shock wave therapy caused redness of the skin and minor superficial bleeding; however, fifteen minutes after the therapy, all animals had recovered from the anesthesia, were using the treated leg, and behaved normally. During the experimental period, no animals showed weight loss. The average weight gain during this forty-nine-day study period was 63 g (range, 54 to 73 g).

MPH-SPECT Scanning

Extracorporeal shock wave therapy induced an increased uptake of ^{99m}Tc-MDP in the treated tibiae compared with the untreated, control tibiae. At two days after the extracorporeal shock wave therapy, the treated legs showed a non significant increase in uptake of ^{99m}Tc-MDP. At seven days, the increase in the metaphysis was more than twofold compared with the uptake in the untreated legs. At twenty-one and forty-nine days, the difference in uptake between the treated and untreated legs had decreased, although at forty-nine days the treated legs still showed 20% more uptake compared with the uptake in the untreated legs (Fig. 2).

At two days after extracorporeal shock wave therapy, the uptake of ^{99m}Tc-MDP in the diaphysis showed a nonsignificant increase in the treated legs compared with that in the untreated legs. At seven days, the uptake in the treated legs was more than twice that in the untreated legs. This highly increased uptake was also

observed at twenty-one days. At forty-nine days, the treated legs still showed 63% more uptake than the untreated legs (Fig. 2).

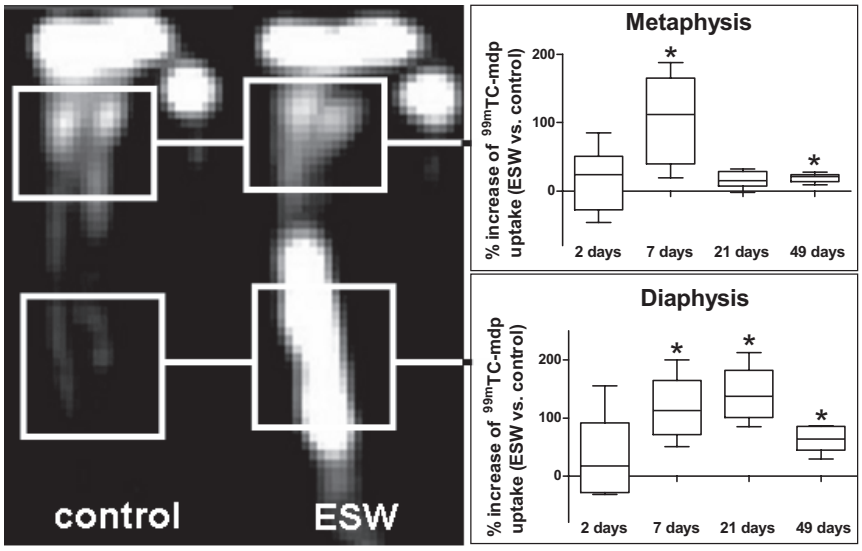

Fig. 2 Representative SPECT scans, made at twenty-one days, of a control tibia and a tibia treated with extracorporeal shock waves (ESW). The regions of interest are indicated and are attached to box-and-whisker plots in which the uptake of technetium-labeled methylene diphosphate (^{99m}TC-mdp) in the treated tibiae is represented relative to the uptake in the controls at different time points. *P < 0.05. The boxes represent the 5% to 95% confidence intervals, the horizontal lines within the boxes represent the median, and the I bars represent the minimum and maximum values.

Microarchitectural Bone Changes

The trabecular volume fraction of the treated legs increased from 20.5% ± 5.55% (mean and standard deviation) at day 0 to 25.3% ± 6.77% at day 49 (p = 0.01) (Fig. 3, a), whereas the trabecular volume fraction of the untreated, control legs (20.3% ± 4.8% at day 0 and 20.1% ± 4.37% at day 49; p = 0.8) did not change significantly during this period. At day 49, all treated legs had a significantly higher trabecular bone volume than the untreated legs (p < 0.01).

At day 49, the connectivity density in the treated legs was 41.9 ± 14.1 compared with 27.7 ± 6.4 in the untreated, control legs (p = 0.02). There was a difference between the structure model index in the treated legs (1.9 ± 0.28) and that in the untreated legs (2.1 ± 0.21) (p = 0.03), indicating that the trabeculae of the treated legs were more plate-like than those of the untreated, control legs. No significant difference in trabecular thickness was found between the treated and untreated legs.

The cortical volume of the treated legs increased from 20.7 ± 0.77 mm^3 at day 0 to 23.9 ± 1.66 mm^3 at day 49 (Fig. 3, b), whereas the cortical volume of the untreated, control legs increased from 20.3 ± 0.83 mm^3 to 21.9 ± 1.34 mm^3 during this period. At day 49, the cortical volume of all treated legs was higher than that of the control legs ($p < 0.0001$). The mean three-dimensional thickness of the cortex increased from 678 ± 39 mm to 767 ± 78 mm in the treated legs ($p < 0.01$) and increased from 670 ± 43 mm to 714 ± 52 mm in the untreated legs ($p < 0.002$). At day 49, the mean cortical thickness of the treated legs was significantly higher than that of the untreated control legs ($p < 0.01$).

At day 49, measurement of the cortical perimeters showed no significant difference in the mean endosteal perimeter between the treated and untreated legs (8.6 ± 0.69 mm and 8.5 ± 0.68 mm, respectively; $p = 0.8$). The periosteal perimeter in the treated legs was significantly higher than that in the untreated legs (15.49 ± 0.54 mm and 15.06 ± 0.48 mm, respectively; $p = 0.03$), suggesting that new bone formation mainly occurred at the periosteal side of the cortex.

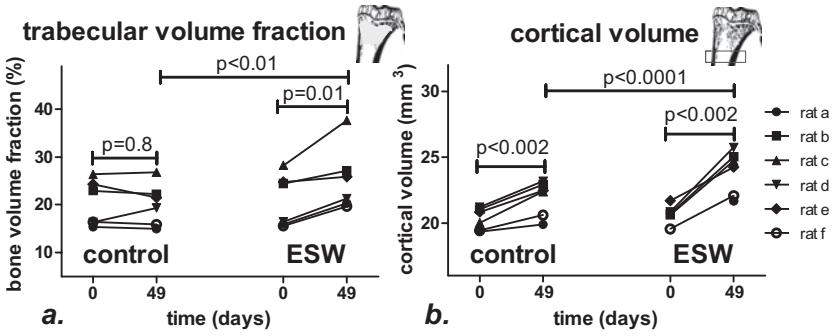

Fig. 3 Trabecular volume fraction (a) and cortical volume (b) for the extracorporeal shock wave (ESW)-treated and control tibiae of all rats. The p values for the differences between the treated and untreated tibiae at day 49 and the p values for the differences between day 0 and day 49 are indicated.

Mechanical Testing

Three-point bending tests showed that the stiffness of the treated tibiae was significantly higher than that of untreated, control tibiae (137 ± 13.2 N/mm and 120 ± 6.7 N/mm, respectively; $p = 0.03$) (Fig. 4, a). The Young modulus did not differ significantly between the treated and untreated legs (1.7 ± 0.2 GPa and 1.8 ± 0.2 GPa, respectively; $p = 0.3$), suggesting that the difference in geometry and increased cortical bone volume explain the increased stiffness in the treated legs (Fig. 4, b). The maximal force did not differ significantly between the treated and untreated legs (101.7 ± 7.3 N and 94.2 ± 7.3 N, respectively; $p = 0.07$) (Fig. 4,

c), and no significant difference in toughness was found between the treated and untreated tibiae (51.4 ±8.8 N mm and 47.0 ± 5.4 N mm, respectively; p = 0.3) (Fig 4, d). There was also no difference in the fracture location. All tibiae fractured at the site of the loading application.

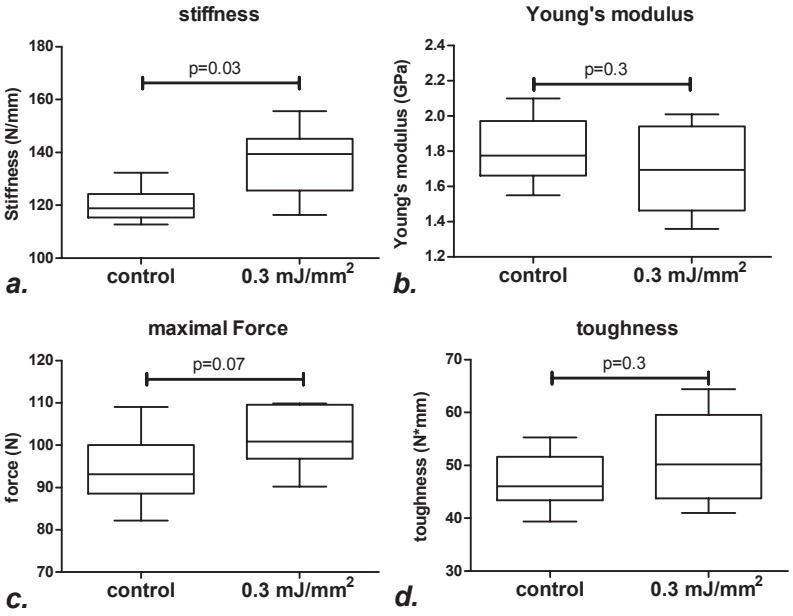

Fig. 4 Box-and-whisker plots of the stiffness, Young modulus, maximal force, and toughness in the control and extracorporeal shock wave-treated (0.3 mJ/mm^2) tibiae. The boxes represent the 5% to 95% confidence intervals, the horizontal lines within the boxes represent the median, and the I bars represent the minimum and maximum values.

Bone Changes on microCT Scans and Histological Analysis.

Examination of the microCT scans showed that the region of the proximal metaphysis that was filled with trabecular bone was larger in the treated legs (an average of 5.8 mm from the epiphysis) than in the untreated, control legs (4.2 mm) (Fig. 5). Furthermore, mineralization in the midpart of the diaphysis was observed in the treated legs (Fig. 5, d). Histological examination showed that these mineralizations were present around fibrotic tissue and had an osseous morphology with osteocytes, suggesting *de novo* bone formation (Fig. 6, a). Active osteoblasts and osteoid were observed in these ossifications (Fig. 6, b). In the cortex, cement lines at the periosteal site, indicating periosteal apposition, could be identified (Fig. 6, c). The periosteum had a normal morphology at forty-nine days (Fig. 6, d).

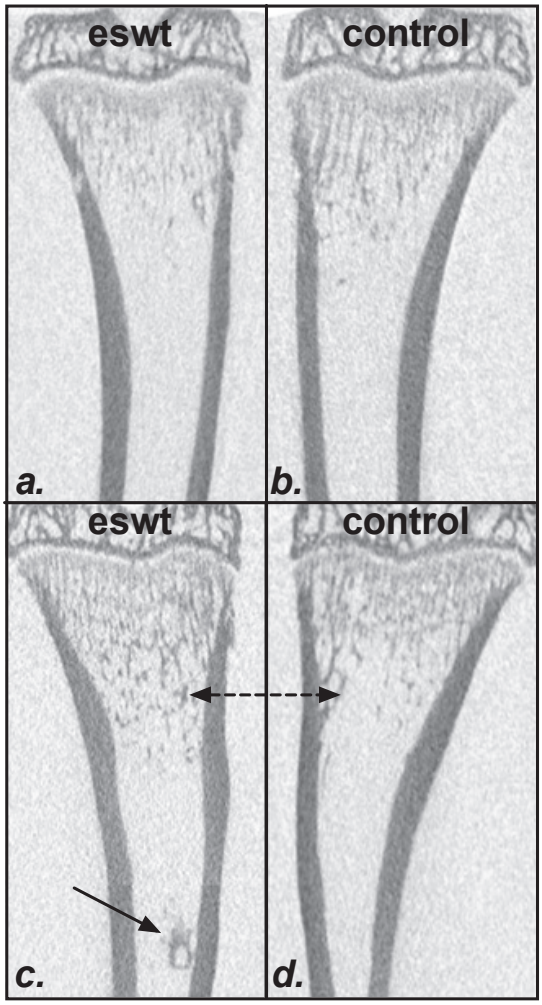

Fig. 5 MicroCT scans prior to (a and b) and forty-nine days after (c and d) extracorporeal shock wave therapy in the same treated (eswt) and same control tibiae. Note the trabecular structures (dashed arrows) and the mineralization in the treated tibia (solid arrow).

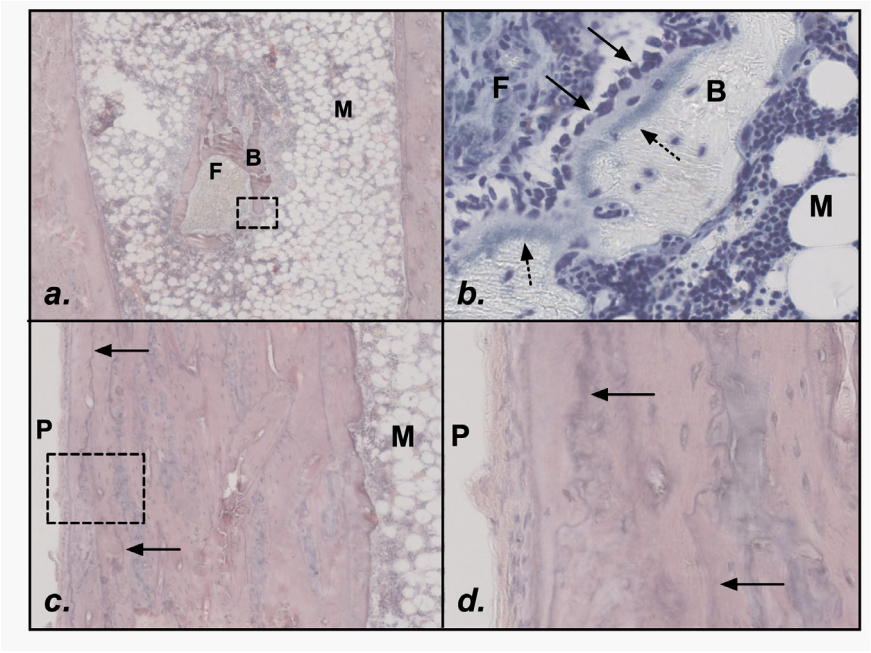

Fig. 6 a: H & E staining of bone (B) in the bone marrow (M) around fibrous tissue (F) (magnification, x5). b: Active bone formation with active osteoblasts (solid arrows) and osteoid (dashed arrows) at the surface of mature bone that contains osteocytes (B). F = fibrous tissue, and M = bone marrow (magnification, x20). c (magnification, 10) and d (magnification x40): H & E staining of the cortex with cement lines (solid arrows) at the periosteal site (P). M = bone marrow, b is a magnification of the box in a, and d is a magnification of the box in c.

In the hind legs harvested twenty-four hours after extracorporeal shock wave therapy, necrotic hematopoietic progenitors and cell debris were observed throughout the bone marrow (Fig. 7, a and b). Furthermore, adipocytes were damaged, characterized by the disruption of cell membranes (Fig. 7, a and b). At forty-nine days, hematopoietic progenitors were reestablished in the bone marrow (Fig. 7, c and d). The adipocyte number and size were, however, higher in the treated legs than in the untreated, control legs at forty-nine days (Fig. 7, e and f). The mean number of adipocytes in the treated and untreated legs was 120.4 ± 18.2 and 69.2 ± 25.6, respectively ($p = 0.003$). The adipocytes in the treated legs had a mean diameter of 0.04 ± 0.004 mm compared with 0.02 ± 0.003 mm in the untreated legs ($p = 0.0002$). At forty-nine days, Perls' staining showed deposition of hemosiderin throughout the bone marrow in the treated legs only, suggesting that extracorporeal shock waves induced the bleeding (Fig. 7, e and f).

At twenty-four hours after extracorporeal shock wave therapy, there were no features of subperiosteal detachment, subperiosteal hemorrhage, or other signs of periosteal damage in the treated tibiae (Fig. 8, a and b).

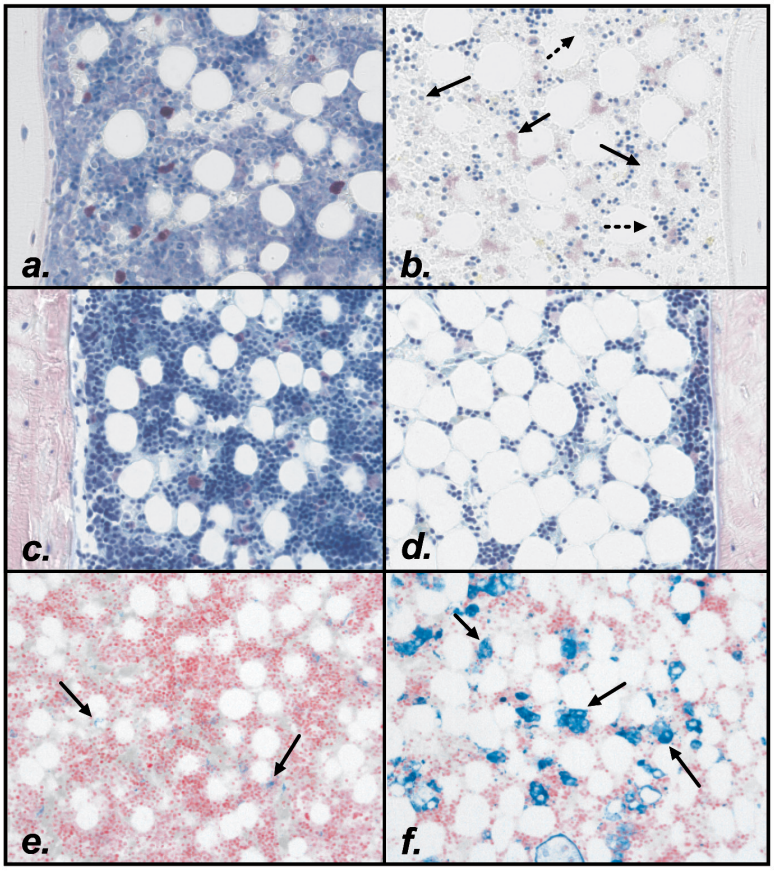

Fig. 7 a and b: May-Grünwald-Giemsa staining of sections of the bone marrow of an untreated control tibia (a) and an extracorporeal shock wave-treated tibia (b) at twenty-four hours after extracorporeal shock wave treatment (magnification, x40). Note the lysis of hematopoietic cells (solid arrows) and the lack of cell membranes around adipocytes (dashed arrows) in the treated leg. c and d: May-Grünwald-Giemsa staining of bone marrow sections of an untreated control tibia (c) and an extracorporeal shock wave-treated tibia (d) at forty-nine days (magnification, x40). Note the hypertrophy of adipocytes. e and f: Perls' staining of sections of an untreated control tibia (e) and an extracorporeal shock wave-treated tibia (f) at forty-nine days (magnification, x20). Note the increased presence of hemosiderin (stained blue and indicated by arrows).

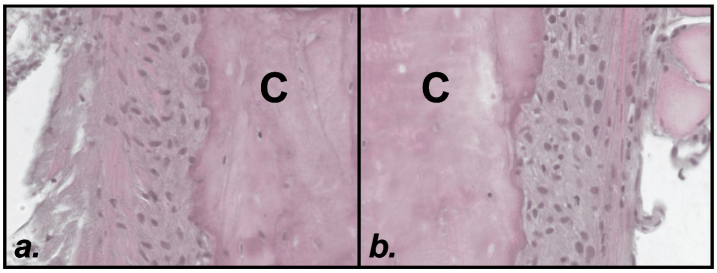

Fig. 8 H & E staining of sections of the cortex of a control tibia (a) and an extracorporeal shock wave-treated treated tibia (b) at twenty-four hours after treatment. Subperiosteal bleeding and periosteal detachment were not observed. C = cortical bone.

Microcrack Analysis

No differences were found between the treated and untreated tibiae in terms of the microcrack parameters of the trabecular bone samples harvested twenty-four hours after extracorporeal shock wave therapy (Fig. 9). The average crack length was 25.1 ± 4.7 µm in the treated legs and 26.9 ± 3.4 µm in the untreated legs (p = 0.5). The average crack density was 0.57 ± 0.27 crack/mm^2 in the treated legs and 0.69 ± 0.29 crack/mm^2 in the untreated legs (p = 0.6). The average crack surface density was 14.8 ± 7.9 µm/mm^2 in the treated legs and 18.2 ± 7.1 µm/mm^2 in the untreated control legs (p = 0.6). No microcracks could be detected in the cortical bone.

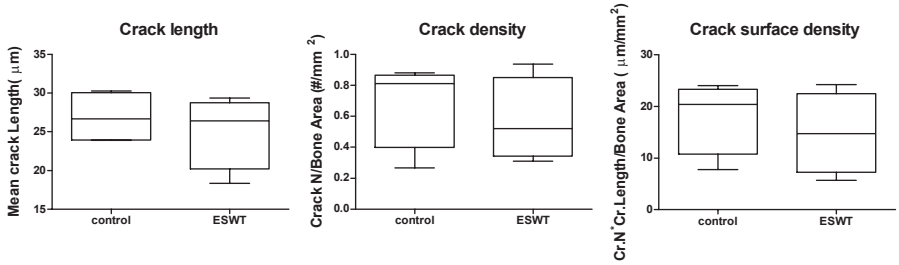

Fig. 9 Results of the microcrack analysis of the control and extracorporeal shock wave-treated (ESWT) tibiae. The mean crack length, crack density, and crack surface density are represented by box-and-whisker plots. The boxes represent the 5% to 95% confidence intervals, the horizontal lines within the boxes represent the median, and the I bars represent the minimum and maximum values.

DISCUSSION

The present study showed that unfocused extracorporeal shock waves induce anabolic effects in cancellous and cortical rat bone. Use of unfocused extracorporeal shock waves led to bone-marrow damage followed by increased bone formation, increased trabecular bone volume, thicker cortices, formation of *de novo* trabecular structures, and an increased amount of adipocytes in bone marrow.

Bone formation was followed over time with use of *in vivo* SPECT scanning of two regions of interest: the metaphysis (consisting mainly of cancellous bone) and the diaphysis (consisting mainly of cortical bone). In both regions of interest, an increased uptake of ^{99m}Tc-MDP following extracorporeal shock wave therapy was found. The greatest increase of ^{99m}Tc-MDP uptake at the metaphysis occurred between two and twentyone days after the shock wave therapy, whereas the uptake at the diaphysis was more pronounced at forty-nine days.

Other investigators who examined the effects of extracorporeal shock waves on bone turnover reported contradictory findings[11, 26, 27]; however, because the authors of these latter studies used focused extracorporeal shock waves, different animal models, and conventional bone scintigraphy, it is difficult to make comparisons with the present study.

At forty-nine days after extracorporeal shock wave therapy, our microCT analysis showed that extracorporeal shock waves induced new bone formation, resulting in increased trabecular volume fraction, increased connectivity, and more plate-like trabeculae. Furthermore, the trabeculae in the treated tibiae continued to be more distal than those in the control legs, suggesting that extracorporeal shock wave therapy also affected bone remodeling in the distal part of the metaphysis, where trabeculae are normally resorbed during growth and aging.

CT reconstructions showed ossifications and *de novo* bone formation in the bone marrow of the diaphysis of all treated legs, whereas no ossification was present in the diaphyseal bone marrow of the untreated, control legs. Because the increased uptake of ^{99m}Tc-MDP in the diaphysis was mainly at the center, it is likely that the increased uptake reflects these ossifying structures rather than bone formation at the cortices. Histologically, the ossifications were characterized by a mineralized matrix with osteocytes. Along the surface, osteoid with active osteoblasts was found, without cartilaginous tissue, indicating intramembranous bone formation.

Treatment with unfocused extracorporeal shock waves induced damage to the bone marrow, resulting in lysis of hematopoietic cells, destruction of adipocytes, and disruption of microvessels. At forty-nine days, the hematopoietic cells had recovered; however, adipocyte hyperplasia and hypertrophy were still present, probably as a result of the bone-marrow injury. The bone-marrow damage might

be related to the finding of fibrous tissue and bone formation. The bone formation always matched with sites of fibrosis. However, from the current findings, it cannot be deduced if (or how) the fibrosis triggers the bone response. We hypothesize that the fibroblasts function as precursor cells and transdifferentiate to osteoblasts, similar to findings reported in distraction osteogenesis[28, 29].

Since microfractures trigger an anabolic response of bone after extracorporeal shock wave therapy, we specifically analyzed the presence of microfractures after the therapy[8, 10, 20]. However, no differences between the treated and untreated legs were found, making it unlikely that microfractures are responsible for the biological responses.

Periosteal detachment and subperiosteal hemorrhages have been described in studies of focused extracorporeal shock waves[10, 20, 30]. We noted no evidence of periosteal damage after use of unfocused extracorporeal shock waves. This finding is in line with those of a study of the effect of energy flux densities of focused extracorporeal shock waves on bone[20]. Periosteal detachment was found with use of energy flux densities of ≥0.5 mJ/mm^2, but not with an energy flux density of 0.35 mJ/mm^2. The current study demonstrated that extracorporeal shock wave therapy can induce an anabolic response to the cortical bone without damaging the periosteum. In a previous study, human periosteal cells subjected to low-energy extracorporeal shock waves showed increased proliferation and calcium deposition and a higher viability than cells that received highenergy extracorporeal shock waves[31].

The present study had several limitations. First, it would have been better if more sequential *in vivo* microCT scans had been made. Future studies should focus on application in an osteoporosis model, evaluate the response of different treatment protocols (different energy flux densities, different numbers of extracorporeal shock waves, different numbers of treatments, etc.), and further elucidate extracorporeal shock wave-induced adipogenesis and other potential side-effects.

Although unfocused extracorporeal shock waves with an energy flux density of 0.1 mJ/mm^2 can be clinically applied without use of analgesics[32], those with an energy flux density of 0.3 mJ/mm^2 can be painful. Therefore, after we had treated the first two animals, we began giving analgesics prior to extracorporeal shock wave therapy. Intravenous analgesia with nonsteroidal anti-inflammatory drugs prior to extracorporeal shock wave therapy for kidney stones has been reported to provide good suppression of pain[33]. Extracorporeal shock wave therapy itself has an analgesic effect, so the gradual increase of the energy flux density during treatment might also help to reduce pain[34, 35].

In conclusion, a single treatment with unfocused extracorporeal shock waves can increase bone turnover and improve the cancellous and cortical bone architecture as well as the mechanical properties of the treated area. In this experimental setup,

extracorporeal shock waves caused damage in the bone marrow, which resulted in hyperplasia and hypertrophy of adipocytes in the bone marrow but did not induce microfractures in the bone or periosteal damage. Further research is needed to explore potential side effects and the clinical implementation of extracorporeal shock wave therapy for osteoporosis. The anabolic response in both cancellous and cortical bone suggests that unfocused extracorporeal shock waves can potentially be used for local treatment of low bone mass and osteoporosis.

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07

Unfocused extracorporeal shock waves induce anabolic effects in osteoporotic rats

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ABSTRACT

Unfocused extracorporeal shock waves (UESW) have been shown to have an anabolic effect on bone mass. Therefore we investigated the effects of UESW on bone in osteoporotic rats with and without anti-resorptive treatment. Twenty week old rats were ovariectomized (n=27). One group was treated with saline and another group with Alendronate 2.4 μg/kg, 3x/week. UESW were applied two weeks after ovariectomy. Thousand UESW were applied to one hind leg, the contra-lateral hind leg was not treated and served as control. With *in vivo* microCT scanning it was shown that in saline treated rats trabecular bone volume fraction (BV/TV) was higher at 2 weeks follow-up in UESW treated legs compared to control legs. However, at 4 and 10 weeks no difference was found. In Alendronate treated animals UESW led to a pronounced anabolic response resulting in an increase in BV/TV at all time-points. Furthermore, UESW resulted in increased cortical volume, higher trabecular connectivity and more plate-like and thicker trabeculae. Biomechanical testing showed that UESW lead to a higher maximum force before failure and higher stiffness in all treatment groups. With histology abundant areas of intramembranous bone formation along the periosteal cortex and within the bone marrow were observed. In conclusion this study shows promising results for the use of UESW in the treatment of osteoporosis, especially when this treatment is combined with an anti-resorptive treatment.

INTRODUCTION

Osteoporosis is a disease characterized by low bone mass and a deterioration of bone microarchitecture leading to an increased fracture risk. Today's standard treatment aims at reduction of further bone loss using anti-resorptive therapy[1]. However, increasing bone mass by anabolic treatment might be more optimal for reducing fracture risk. To date anabolic treatment is limited to the use of recombinant parathyroid hormone or its analog. This treatment requires daily subcutaneous administration, accompanies potential dangerous side effects like hypocalcaemia, and is expensive. Therefore this therapy is limited to patients that do not respond to or have contraindications for bisphosphonates[1].

As an alternative to pharmacologic treatment biophysical stimuli have been suggested, but mechanical vibration, pulsed electromagnetic fields and ultrasound have so far not been proven to be beneficial in osteoporosis[2-5]. Previously we showed that unfocused extracorporeal shock waves (UESW) with an energy flux density (EFD) of 0.3 mJ/mm^2 can also induce anabolic effects in healthy rat bone[6]. Pronounced increased bone formation was observed in the cortical bone and the bone marrow seven days after UESW were applied to the hind leg of healthy male rats. This resulted in increased trabecular and cortical bone mass and improved biomechanical properties. Furthermore, we have shown that UESW can beneficially affect bone microarchitecture in a rat osteoporosis model[7]. These experiments were however done with a lower EFD (0.16 mJ/mm^2) and we did not find an anabolic bone response.

So far, most research included focused extracorporeal shock waves. These shock waves are used in a variety of musculoskeletal disorders like nonunions and delayed unions, diaphyseal fractures, stress fractures, osteonecrosis of the femoral head, Achilles tendinopathy and fasciitis plantaris[8-12]. Shock waves are acoustical pulses with a high amplitude (+-100 bar) and a short rise time (≤10 ns) in the frequency spectrum between 16 Hz and 12Mhz[13]. In focused shock wave therapy the waves converge in a focal point. In contrast, unfocused shock waves are produced as a parallel bundle, enabling a homogenous treatment of larger regions.

In experimental studies examining the effects of focused shock waves on bone it has been shown that a single treatment led to an increased differentiation of bone marrow stem cells towards osteoprogenitor cells[14, 15]. Furthermore, it has been shown that several growth factors that are important for bone regeneration, including VEGF, TGF-beta 1 and several BMPs, are upregulated after extracorporeal shock wave treatment[14, 16-18].

To explore the potential use of UESW to increase bone mass and subsequently reduce fracture risk in osteoporotic patients, we examined the effects of UESW on the bone microarchitecture and biomechanical properties in a rat model for osteoporosis. To investigate the additional clinical value of UESW in the presence of an anti-resorptive treatment with bisphosphonates, conditions with and without Alendronate (Fosamax) were examined.

METHODS

Animals

27 Female Wistar rats, age 20 weeks, were obtained (Harlan Laboratories, Horst, the Netherlands). The animals were housed in threesomes at the animal facility of the Erasmus MC, with a 12 h light-dark regimen, at 21°C and received standard food pellets and water ad libitum. The research protocol (116-08-02/EUR 1455) was approved by the local committee for Animal Experiments and is in accordance with Dutch law.

Three groups of 6 animals were used for evaluation of bone changes during a 10-week follow-up period with *in vivo* microCT scanning and these rats were used for biomechanical testing. The remaining 9 animals were used for histological and ex-vivo microCT evaluation only and were not included in the statistical analysis. Of these nine animals, three animals were euthanized 24 hours after UESW were applied (no Alendronate) and 6 animals were euthanized at 1 week after UESW were applied (3 received saline and 3 received Alendronate).

Research model

All rats received a bilateral ovariectomy (OVX) to simulate osteoporosis. This procedure was performed under sterile conditions using gas anesthesia (oxygen with 2% isoflurane; Rhodia Organique Fine Ltd., Bristol, UK). Buprenorfine (Schering-Plough, Kenilworth, NJ, USA), 0.05 mg/kg/12 h, was given for two days. Three treatment groups of 6 animals were made. The first group received subcutaneous injections with saline three times per week, starting the first day after OVX. The second group received subcutaneous injections with Alendronate (ALN), 2.4 μg/kg, 3x/week. This is comparable to a human dose of 70 mg/week[19]. Treatment was also started the first day post-operatively. In these two groups UESW were applied 2 weeks after OVX. The third group received subcutaneous saline injections similar to the first group, but UESW treatment was applied 2 days after OVX was performed. Because UESW were applied a short time after OVX the bone volume

at time of UESW treatment is comparable to the Alendronate treated group, in which little bone loss is observed.

Unfocused shock wave therapy

One hind leg was treated with UESW, the contra-lateral hind leg was not treated and served as control. Under gas anaesthesia with oxygen and 2% isoflurane both hind legs were shaved and an ultrasonic gel that served as coupling media was applied. The shock wave applicator was placed at the antero-lateral side of the hind leg, such that he whole tibia was covered. A total of one thousand electro-hydraulically generated UESW were applied at 3 Hz with an energy flux density (EFD) of 0.3 mJ/mm^2 (Dermagold, Tissue Regeneration Technologies, Woodstock, GA, USA, manufactured by MTS, Konstanz, Germany).

In vivo microCT scanning

At 0, 2, 4 and 10 weeks after UESW treatment microCT scans were made under gas anaesthesia (isoflurane/oxygen). The proximal 17 mm of the tibia was scanned (60 kV, 167 μA, using a 0.5 mm aluminium filter, over 196º with a rotation step of 1º), which takes 8 minutes (Skyscan 1076 scanner, Kontich, Belgium). This resulted in datasets with an isotropic voxel size of 18 μm. Three-dimensional (3D) reconstructions of two regions of interest were made (NRecon software, Skyscan). The first is of the proximal metaphysis of which trabecular bone parameters were calculated (starting just distal of the epiphysial growthplate and continuing until 2.7 mm more distal). The second reconstruction was from the diaphysis. On these reconstructions cortical bone parameters were determined (starting 9 mm distal from the epiphysis and continuing until 3.6 mm more distal). Bony and non-bony structures were separated using a local threshold algorithm (software freely available)[20] resulting in binary datasets[21]. Cortical and trabecular bone were automatically separated using in-house software. Trabecular architecture in the proximal metaphysis was characterized by determining trabecular volume fraction (BV/TV), connectivity density (Conn/TV), structure model index (SMI), and 3D trabecular thickness (TbTh). Cortical architecture in the diaphysis was characterized by cortical volume (CtV) and cortical thickness (CtTh).

Three-point bending tests

Mechanical properties were determined using a three-point bending test. At 10 weeks after UESW, animals were euthanized using an overdose of pentobarbital. The tibiae were dissected, soft tissue removed, and stored in PBS at 4°C. Testing was performed with the bones equilibrated to room temperature. Each tibia was stably positioned on its medial antero-lateral site. The distance between the

loading posts was 2.2 cm, in this way the posts were just distal from the condyles and just proximal from the maleoli. To ensure a stable position of the bone five pre-conditioning runs with a displacement rate of 0.01 mm/s and a maximal force of 4 N were done (Single Column Lloyd LRX Testing System, Lloyd Instruments, Hampshire, UK). Hereafter a displacement rate of 0.01 mm/s was applied until fracture of the tibia. Displacement (mm) and force (N) were registered at a sample rate of 20 Hz. Force displacement graphs were made and load to failure and stiffness were determined.

Histology

The tibiae were fixed in 4% paraformaldehyde. The tibiae hind legs used for mechanical testing at 10 weeks after UESW treatment were directly fixed after testing. After 21 days of fixation the proximal half of the tibiae were dehydrated and block embedded in methyl methacrylate (MMA). Sagittal sections (6 μm thick) were made. Histological assessment (e.g. new bone formation, marrow status and cell characterization) was evaluated using haematoxilin-eosin and thionine staining (0.05% thionine in 0.01 M aqueous sodium phosphate, pH 5.8 for 5 min).

Statistics

All parameters were statistically analyzed for each time point using paired t-tests, in which the UESW treated and untreated control legs served as a pair (GraphPad Software, San Diego, CA, USA). Changes in BV/TV and CtV at each time point relative to baseline were statistically tested in treated and untreated legs separately, for each treatment group, using mixed model regression (SPSS 16, Chicago, USA). Differences of biomechanical parameters of treated legs between the three treatment groups were statistically analyzed with one-way ANOVA with a Tukey's multiple comparison test.

RESULTS

In vivo microCT analysis

In saline treated rats receiving UESW 2 weeks after OVX, BV/TV was 22.2% (SD 3.3) at baseline (t=0). During 2 week follow-up trabecular bone loss as a consequence of OVX was much lower in UESW treated legs than in non-treated control legs (Fig. 1a). At 2 weeks BV/TV was 20.2% (SD 3.5) in UESW treated legs compared to 13.8% (SD 3.0) in untreated control legs (p=0.003). This demonstrates that in UESW treated legs the cancellous bone volume was 46% higher compared to untreated controls. However, at 4 and 10 weeks no difference could be found.

Morphometric analysis of the cancellous bone showed more plate-like trabeculae (lower SMI), thicker trabeculae and a higher trabecular connectivity in UESW treated legs compared to untreated control legs at 2 weeks after UESW (Table 1). At 4 and 10 weeks no difference between treated and untreated legs were found.

Cortical volume was significantly higher in UESW treated legs than in control legs during follow-up at all time-points (Fig. 1b). The cortical volume in UESW treated legs increased with 8.0% (SD 2.9), 13.4% (SD 5.5), and 13.0% (SD 6.2) relative to baseline at 2, 4 and 10 weeks respectively. This was less in untreated control: 1.3% (SD 2.0), 3.7% (SD 1.5) and 3.2% (SD 3.3) at 2, 4 and 10 weeks respectively. Cortical thickness was also higher in UESW treated legs compared to control legs at 2, 4 and 10 weeks (Table 1).

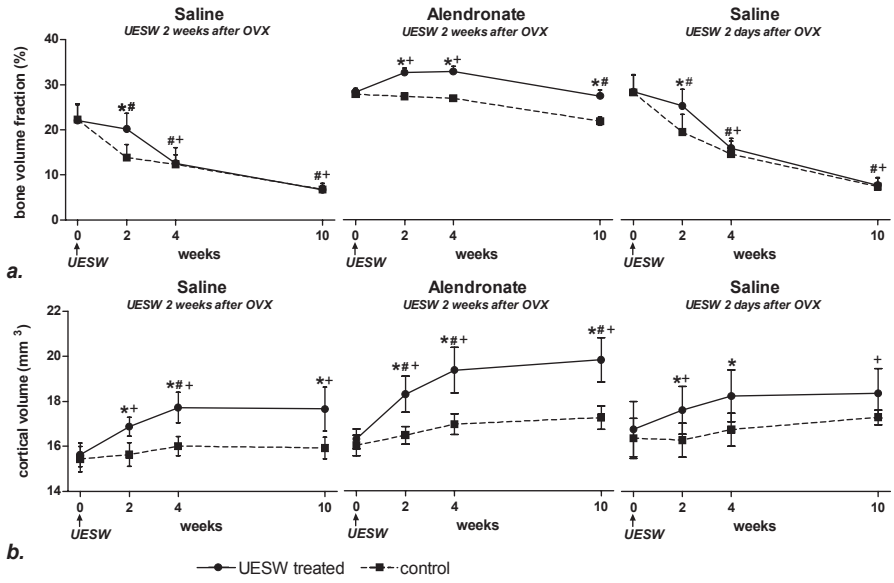

Fig. 1 Trabecular bone volume fraction (BV/TV) (a) and cortical bone volume (b) determined by *in vivo* microCT scanning. * indicaties p<0.05 analyzed with paired t-test a specific time-point. # indicates p<0.05 for controls compared to t=0 and + indicates p<0.05 for treated legs compared to time-point t=0 (mixed model statistics).

In rats receiving Alendronate, BV/TV was 28.2% (SD 1.6) at baseline. UESW resulted in an increase in BV/TV during follow-up, which resulted in a significantly higher BV/TV compared to untreated contralateral legs at 2, 4 and 10 weeks after UESW (2 weeks: 32.8% (SD 2.6) vs 27.4% (SD 1.6) (p=0.0002), 4 weeks: 33.0% (SD 2.8) vs 27.0% (SD 1.0) (p=0.0003) and 10 weeks: 27.5% (SD 3.3) vs 21.9% (SD

2.2) (p=0.0002) (Fig. 1a). In UESW treated legs the cancellous bone volume was 20, 22 and 26% higher at 2, 4 and 10 weeks respectively. After UESW treatment trabeculae were more plate-like (lower SMI), thicker and had a higher connectivity at all time-points. (Table 1).

Cortical volume was significantly higher in UESW treated legs compared to control legs during follow-up (Fig. 1b). Cortical volume increased with 12.2% (2.7), 18.7% (4.5) and 21.5% (3.2) from baseline at 2, 4 and 10 weeks, respectively. In control legs this was 2.8% (SD 1.7), 5.9% (SD 2.0) 7.7% (SD 2.8) at 2, 4 and 10 weeks respectively. Cortical thickness was also higher in UESW treated legs at all time points during follow-up (Table 1).

In the third group, in which UESW were given 2 days after OVX, BV/TV was 28.4% (SD 3.7), similar to the Alendronate treated group. UESW did again result in reduced trabecular bone loss after UESW treatment with a BV/TV of 25.3% (SD 3.6) in UESW treated legs compared to 19.5% (SD 3.9) in untreated control legs (p=0.003) at 2 weeks follow-up (Fig. 1a). This demonstrates a 30% higher cancellous bone volume in the treated legs compared to untreated controls at 2 weeks. At 4 and 10 weeks no difference in BV/TV was found. At 2 weeks, but not at 4 and 10 weeks SMI was lower and connectivity density and mean trabecular thickness were higher in UESW treated legs compared to controls (data not shown). UESW again resulted in higher cortical volume and cortical thickness in UESW treated legs compared to untreated controls (Fig. 1b).

Table 1 Bone parameters determined with *in vivo* microCT scanning

	Saline							Alendronate						
	wk 0	**week 2**		**week 4**		**week 10**		**wk 0**	**week 2**		**week 4**		**week 10**	
		UESW	***control***	***UESW***	***control***	***UESW***	***control***		***UESW***	***control***	***UESW***	***control***	***UESW***	***control***
trab.th. (μm)	104.2 (3.7)	107.0* (2.6)	96.5 (2.5)	111.1 (9.7)	101.1 (4.1)	103.6 (4.4)	99.3 (2.8)	109.4 (2.8)	115.3* (4.4)	105.6 (2.4)	117.4* (6.3)	105.3 (1.5)	112.7* (7.4)	101.0 (4.3)
conn.dens.	47.9 (12.2)	38.9* (11.8)	20.3 (9.0)	11.7 (1.5)	15.4 (6.0)	1.5 (2.7)	2.4 (3.1)	76.4 (4.6)	89.7* (7.7)	76.7 (5.7)	87.5* (5.3)	74.3 (4.4)	64.6* (8.1)	47.8 (9.0)
SMI	2.4 (0.2)	2.3* (0.2)	2.6 (0.1)	2.7 (0.2)	2.6 (0.1)	2.8 (0.1)	2.8 (0.1)	1.9 (0.1)	1.7* (0.1)	2.0 (0.1)	1.7* (0.1)	2.0 (0.1)	2.1* (0.1)	2.3 (0.1)
cort. thick. (μm)	658.6 (30.0)	692.7* (14.2)	666.7 (31.8)	725.2* (12.9)	683.6 (25.1)	726.0* (21.7)	684.6 (24.5)	646.7 (25.1)	699.9* (32.3)	652.6 (20.7)	750.6* (34.9)	680.2 (22.7)	773.5* (34.9)	693.2 (18.2)

Mean with standard deviation. * indicates $p<0.05$ in UESW treated compared to untreated controls at the same time point. trab.th. = mean trabecular thickness, conn.dens. = connectivity density, SMI = structure model index, cort.thick. = cortical thickness.

Mechanical testing

In both the saline and the Alendronate group UESW treated legs showed significant higher maximal force at failure and higher stiffness (Fig. 2). In saline treated rats that received UESW 2 weeks after OVX the maximal force and stiffness were respectively 14 and 10% higher compared to untreated controls. In Alendronate treated rats maximal force and stiffness were respectively 15 and 18% higher and in saline treated rats that received UESW 2 days after OVX they were respectively 10% and 19% higher. Furthermore, maximum force and stiffness were significantly higher in Alendronate treated animals compared to saline treated animals (p=0.02 for maximum force at failure, and p=0.01 for stiffness).

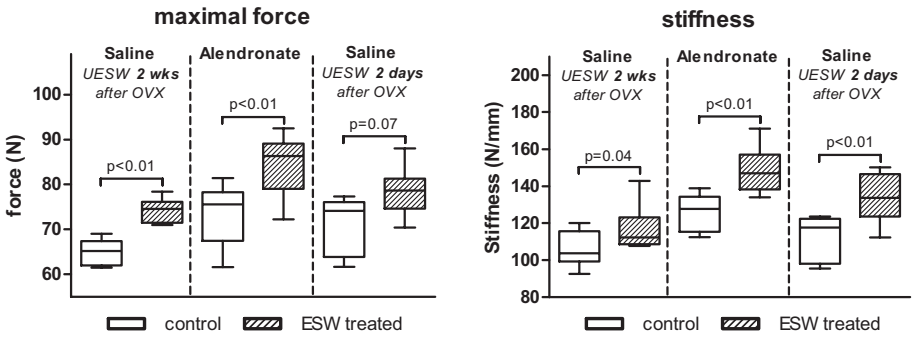

Fig 2 Maximal force (a) and stiffness (b) determined by three point bending test.

Ex vivo microCT analysis and histology

On microCT reconstructions at 1 week after UESW treatment, abundant areas of new bone formation were found (Fig. 3a). These areas were seen along the periosteal cortex and in the bone marrow. The latter was seen distally to where trabeculae normally extend and appears to be *de novo* bone formation. With histology it is demonstrated that in both these areas very active intramembranous new bone formation exists (Fig. 3b). At the cortex many active osteoblasts, osteoid and immature bone are found (Fig. 3c). Areas with hypertrophic chondrocytes and mineralized callus were observed only sparsely. In the cortex of UESW treated tibiae osteocytes with defragmented or pyknotic nuclei and empty lacunae were found. This seemed more pronounced at the endosteal site. No periosteal detachment or subperiosteal haemorrhage were observed. In the bone marrow active osteoblasts, osteoid and immature bone were seen abundantly around fibrotic tissue with fibroblasts (Fig. 3d).

Further, on microCT reconstructions cortical cracks were identified in three of the six animals. The undisplaced fractures were seen in the diaphysis only, running perpendicular to one of the three cortical columns (6 to 10 mm in length). Which column was affected varied. We found no more than one crack per animal. New

bone formation was not directly related to these cracks since this was seen at all cortices (Fig. 4).

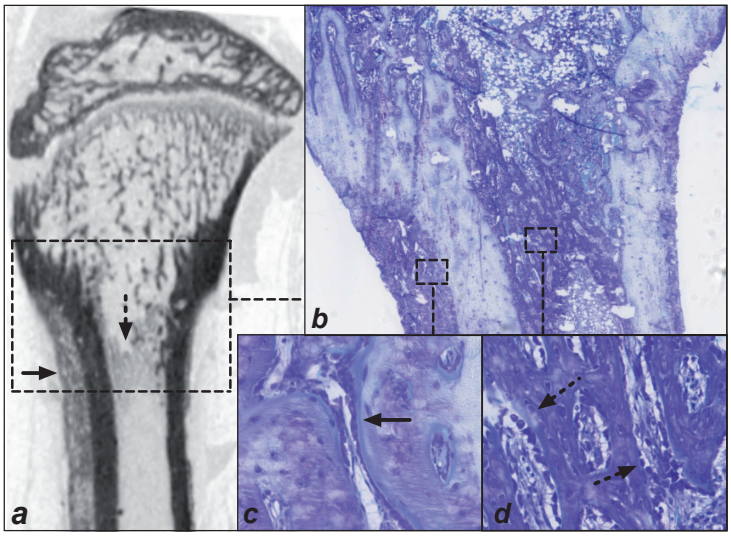

Fig 3 Reconstruction of a microCT scan of a treated tibia at 7 days after UESW were applied. The rat was treated with saline. New bone formation can be seen in the bone marrow (dashed arrow) and at the periosteal cortex (solid arrow) (a). Thionine staining of the area indicated by the dashed box (40x) (b). New bone formation at the periosteal cortex with osteoid and active osteoblasts (solid arrow) (100x) (c). Intramembranous bone formation with abundant active osteoblasts (dashed arrows), inmature bone and fibrous tissue (100x) (d).

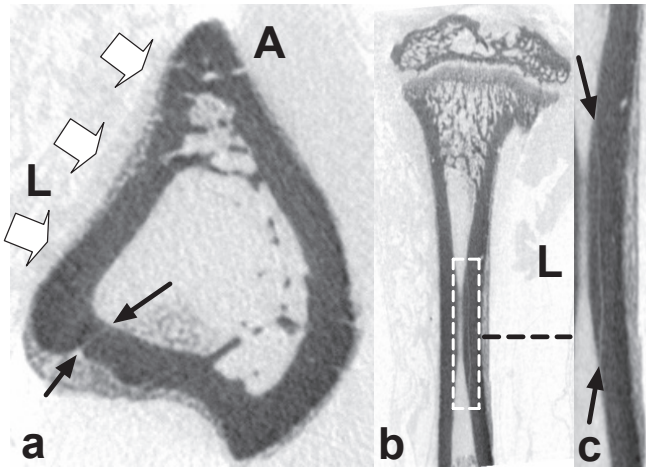

Fig. 4 Reconstruction of an ex-vivo micro CT scan 1 week after UESW treatment. Transversal reconstruction, the direction of the UESW is presented by the open arrows and the black solid arrows indicate is a micro fracture (a). A sagittal reconstruction (b), with in the dotted box an enlargement of the cortical area that has a micro fracture (c), which is indicated by solid black arrows. A = anterior, L=lateral.

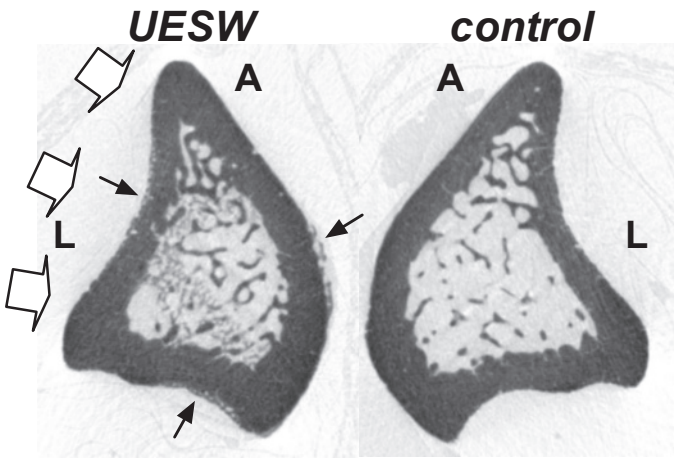

Fig. 5 Reconstruction of an ex vivo microCT scan of an UESW treated and a control tibia at 1 week. The rat was treated with saline. The direction of UESW is shown by the open arrows. New bone formation can be seen at all cortices (solid arrows) and in the bone marrow of the UESW treated tibia. A=anterior, L=lateral.

DISCUSSION

In this study we show that a single treatment with UESW leads to an increase in trabecular and cortical bone volume, which leads to significantly improved biomechanical properties and thus to a theoretical reduction of the fracture risk. Interestingly, these effects were more pronounced in osteoporotic rats receiving Alendronate, a bisphosphonate that is widely used among osteoporotic patients.

Today's standard treatment of osteoporosis is an anti-resorptive treatment with bisphosphonates. Alendronate is widely used in that perspective. To investigate the potential role of UESW for osteoporotic patients, the effect of UESW was investigated in situations with and without treatment with Alendronate. We found that UESW lead to an increase in trabecular bone volume in both saline and Alendronate treated animals. The effect of UESW on trabecular bone in saline treated animals was only transient, while in Alendronate treated animals this increase was preserved during follow-up. Furthermore, the effect of UESW on cortical bone mass was more pronounced in Alendronate treated rats. Alendronate has a direct effect on the activity of osteoclasts, which subsequently leads to inhibition of bone resorption[22]. Although bone remodeling is mostly a coupled system ESW did induce strong anabolic effects, which suggest that bone modeling was stimulated rather than bone remodeling.

To be assured that these favorable effects were due to the anti-resorptive effects of the bisphosphonate and not due to the higher bone volume at time of UESW treatment, we examined a third group. In this saline treated group UESW treatment was applied two days after ovariectomy when BV/TV was comparable to BV/TV in the group that received Alendronate for two weeks after OVX. Again UESW induced an increase in BV/TV, but this effect was not maintained at longer follow-up. This indeed suggests that the anti-resorptive treatment itself and not the amount of trabecular bone at time of UESW treatment was responsible for the increase and preservation of trabecular bone mass after UESW treatment.

Biomechanical testing showed that UESW induced bone changes result in improved mechanical properties which were most pronounced in Alendronate treated animals. This suggests that a treatment with UESW in the presence of an anti-resorptive treatment might further reduce fracture risk.

Abundant areas of new bone formation are already seen 1 week after UESW are applied. It is remarkable to see that the new bone formation at the periosteal site and in the bone marrow was predominantly intramembranous. In line with this is a report in which focused ESW to rat tibiae resulted in cambium cell proliferation and subsequent intramembranous osteogenesis[23]. Furthermore, Takahashi *et al*. demonstrated that treatment with focused ESW lead to an increased expression of genes that are suggested to relate to intramembranous bone formation[24]. In a previous report in which we applied UESW with an EFD of 0.3 mJ/mm^2 to the hind leg of non-osteoporotic male rats, we found that UESW resulted in lysis of hematopoetic cells, destruction of adipocytes and disruption of micro-vessels[6]. We did not observe these features when osteoporotic rats were treated with UESW with an EFD of 0.16 mJ/mm^2 [7]. We believe that in the current study the effects of UESW on the bone marrow led to the formation of fibrotic tissue. Subsequently, extensive areas of intramembranous bone formation are formed. These features show great similarity with bone marrow ablation models. These models are used to examine bone regeneration after the bone marrow is mechanically removed[19]. Regeneration is followed through specific stages including an inflammatory stage with clot formation, a repair phase with neovascularisation and cell migration (including mesenchymal stem cells) and finally a remodelling phase to re-establish the hematopoietic and fat tissue in the bone marrow. Histological images of bone regeneration in bone marrow ablation show great resemblance to our findings. Interestingly, bone regeneration in bone marrow ablation is solely by intramembranous bone formation[25]. This might suggest that treatment with UESW, which also results in intramembranous bone formation, induce similar biological responses. This needs however to be determined in future studies.

In the current study we found cortical fractures after UESW treatment (Fig. 4). This is in contrast to earlier experiments we have performed both at an EFD of 0.16 and 0.3 mJ/mm^2 [2, 3]. Because these cracks were only sporadically induced and varied in location, they might be the result from an indirect effect of the shock-waves, known as cavitation[13, 26]. This indirect effect, can occur when gas bubbles in soft tissue or liquid tissue, in this case blood in the Haversian system, grow by the positive pressure of the shock wave and eventually collapse. During the collapse, energy is released and high mechanical forces occur, which may cause a cortical crack as was also demonstrated in focused shock wave treatment [27, 28]. Since new bone formation was seen in the diaphysis of all cortices, we believe the biological responses are independent of cortical cracks (Fig. 5). Whether these effects can also occur if the same shock waves are applied in larger species is yet unknown.

A disadvantage of (U)ESW is that the application can be very painful, especially when they are produced electro-hydraulically. Although it has been described that treatments with an energy flux density (EFD) up to 0.17 mJ/mm^2 can be applied without additional analgesia[29], we believe that especially in the older, fragile patient additional analgesia is indicated. In that perspective it has been shown that intravenous supply of Paracetamol, NSAIDs or Tramadol has good pain relieving results in ESW therapy for kidney stones[30]. Alternatively, UESW treatment can be applied when the patient receives anaesthesia for other indications, for instance when surgical treatment for osteoporotic fractures is indicated, UESW can be applied to the contra-lateral site and other skeletal regions to prevent other fractures.

The current study shows promising results for the use of UESW in the treatment of osteoporosis. Especially when combined with an anti-resorptive treatment with bisphosphonates, UESW led to increased bone mass and improved biomechanical properties. Since anti-resorptive treatment with bisphosphonates is the standard treatment for osteoporosis, UESW treatment might have important implications for osteoporotic patients. Further reduction of the fracture risk might be achieved by treating those sites that are specifically vulnerable to fracture in osteoporosis. Since several clinical studies used UESW for other modalities, concerns of adverse side effects seem limited. All together these results suggest the need for a clinical study to examine the effects of UESW on bone density in humans. Most relevant skeletal sites to start with include the forearm and proximal femur. Furthermore, more research is needed to investigate the biological causes that lead to the anabolic bone response after UESW are applied.

In conclusion, this study shows that treatment with UESW leads to increased bone mass and improved biomechanical properties especially when treatment is combined with bisphosphonates. This suggests that UESW might further reduce fracture risk and can be of benefit for osteoporotic patients.

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08
Discussion

In the search for a therapy for osteoporosis other than the currently applied pharmaceutical treatment, we examined the effects of several biophysical stimuli on bone microarchitecture in small animal models. We found that unfocused extracorporeal shock waves (UESW) induce pronounced anabolic effects on cancellous and cortical bone, and lead to *de novo* bone formation. These findings indicate that application of UESW is a potential treatment for osteoporosis. In contrast, we found no support for the use of pulsed electromagnetic fields (PEMF) or whole body vibrations (WBV) in the treatment of osteoporosis.

UNFOCUSED EXTRACORPOREAL SHOCK WAVE THERAPY

ESW (extracorporeal shock waves) are widely used for many musculoskeletal disorders, but their effect in osteoporosis has not been examined[1-8]. This might be because ESW therapy has a focal character, or because the pain associated with ESW therapy is a barrier to exploring such treatment in a systemic disease such as osteoporosis.

The focal character of ESW is related to its use in the treatment of kidney stones, in which most experience with ESW therapy has been established. In this setting a small area is treated with a converged focal bundle[9, 10]. With non-focused or unfocused ESW therapy the bundle of shock waves is not converged but is more or less parallel, or even somewhat divergent. With the use of unfocused shock waves, larger skeletal areas can be treated, which also enables application in osteoporosis. Generators that produce this type of unfocused shock waves have recently been developed, in particular for the treatment of wounds[11].

Concerning the pain issue, it is known that the magnitude of the energy flex density (EFD) is related to pain sensation during treatment. When the EFD is kept at 0.16 mJ/mm^2 or lower, application can be made without anesthesia. Moreover, these lower EFD settings can affect bone. In segmental defects, focused ESW with an EFD of 0.16 mJ/mm^2 stimulated bone regeneration, and when healthy rats were treated with focused ESW there was an increase in osteoprogenitors[12-14].

In the first explorative study we used the above-mentioned characteristics as starting point. We examined the effect of unfocused shock waves with an EFD of 0.16 mJ/mm^2 in a rat osteoporosis model as well as in non-osteoporotic animals. Treatment with 2000 UESW resulted in an increased trabecular volume fraction, higher connectivity density, and more plate-like trabeculae in sham-ovariectomized rats during the 7-week follow-up. In ovariectomized rats the trabecular volume fraction was about 10% higher at 7 weeks post ESW treatment with 2000 shock waves, compared to non-treated controls. The magnitude of this effect is comparable to a

medium dose of bisphosphonate[15]. Using more than six animals per group might have yielded a stronger significance level, also in the group that received 2 times 1000 shock waves. However, the magnitude of the effect was small and probably not of clinical relevance. Furthermore, in rats treated 10 weeks after ovariectomy when only 8% of original bone volume/tissue volume (BV/TV) was left at the proximal tibial metaphysis, ESW treatment was not effective. In this study we found no evidence for cortical thickening or *de novo* bone formation. Histological analysis at the end of the experiment revealed no difference in the presence of woven bone or presence of osteoid between UESW-treated legs and controls. Therefore, it seems that UESW with an EFD of 0.16 mJ/mm^2 mainly affects existing trabecular structures. In this case, clinical application might be beneficial in osteopenic rather than in osteoporotic bone.

Since UESW seemed to affect bone turnover, we analyzed this using novel multi-pinhole (mph)-SPECT scanning. However, when we treated the hind leg of healthy rats with unfocused shock waves with an EFD of 0.16 mJ/mm^2 we found no evidence of increased bone turnover during the two week follow-up. In a subsequent attempt to observe an effect, UESW with an EFD of 0.3 mJ/mm^2 was applied. This setting was chosen simply because this was the highest possible dose with the device used (Dermagold, Tissue Regeneration Technologies, USA). This application resulted in a major response in bone formation. At the metaphysis an increased uptake of radiolabeled diphosphonate was shown by mph-SPECT analysis at 7-day follow-up. At the diaphysis the uptake was even more pronounced and remained increased during 7 weeks of follow-up. These findings suggest pronounced bone modeling activity, especially at the diaphysis. Indeed histological analysis showed a high number of active osteoblasts and osteoid in the bone marrow, as well as evidence of cortical thickening. Because of the overwhelming activity on mph-SPECT scans at the diaphysis it was not possible to differentiate between uptake at the cortex and at bone marrow.

The effect of ESW on bone remodeling has also been studied. In horses, conflicting results were found. Focused ESW with an EFD of 0.15 mJ/mm^2 applied to the fourth metacarpal bone led to an increase in uptake of radiolabeled diphosphonate at 3 and 16 days after treatment[16]. However, another study found no effect of this same treatment on bone scintigraphy, and unfocused radial ESW with 0.1 mJ/mm^2 also seemed to have no effect on bone turnover[17, 18]. In rabbits, focused ESW with an EFD of 0.5 or 0.9 mJ/mm^2 induced a decrease in bone turnover and local blood flow, 10 days after treatment[19]. In our study we used mph-SPECT scanning to evaluate the uptake of radiolabeled diphosphonate. The resolution of this method is up to 1 mm and allows to examine changes in bone turnover very precisely in both quantity and location. Comparison with earlier scintigraphy-based studies is

difficult because of differences in the treatment protocols, evaluation methods and the animal models used.

In our study the increased activity found with SPECT scanning in UESW-treated tibiae matched the findings of microCT scanning and histology, i.e. a 25% increase in BV/TV in trabecular bone, increased cortical volume with periosteal thickening, and *de novo* bone formation at the diaphysis were found. Cortical thickening was also observed in rabbits treated with focused shock waves with an EFD of 0.5 mJ/mm^2 [19-21] . The use of higher EFDs also led to bone formation at the periosteal site, but was accompanied by cortical fractures[21].

Using biomechanical testing, our study shows that UESW treatment led to an increased bending stiffness and maximum force before failure. This increased mechanical competence is mainly due to cortical thickening, since the most important parameter in this respect - the moment of inertia - mostly relates to the cortical thickness and perimeter. The fact that we found no difference in the Young's modulus of bone tissue implies that the increased stiffness can indeed be explained by the increase in the moment of inertia.

An important finding in our study was that there was no difference in the presence of microcracks in the trabecular and cortical bone. It was often assumed that shock waves induce micro-damage to the bone, which subsequently leads to a process of bone regeneration with a net bone gain [20, 22, 23]. Shock waves can lead to cortical fractures; in a dog study this phenomenon appeared to be dose-dependent[22]. These fractures occur when about 3000 ESW with an EFD of 0.23 mJ/mm^2 are applied, or after about 1400 ESW with an EFD of 0.54 mJ/mm^2. However, bone-stimulating effects have been demonstrated with lower EFDs and a lower number of pulses[12, 13]. Our data with unfocused shock waves confirm a clear osteogenic effect without inducing osseous damage when 1000 shock waves at an EFD of 0.3 mJ/mm^2 were applied to rat tibia.

Another important finding was the observation of *de novo* bone formation after UESW treatment. This new bone tissue appeared in the bone marrow and was surrounded by fibrotic tissue and not attached to any existing bone surface. The finding of hemosiderin deposition, lysis of hematopoietic precursors, cell debris and disruption of cell membranes of adipocytes suggest that UESW caused overall damage to the bone marrow. Indications that high-dose focused ESW could induce intramedullary damage (including bleeding, bone marrow edema and trabecular disruption) has been shown in rabbits[19, 21]. These effects are thought to be the consequence of cavitation, which is the effect of gas bubbles being introduced in blood and other fluids after ESW are applied. Subsequently, these gas bubbles gain in size and energy due to the negative tensile of the shock wave and, on collapse, can cause severe damage[24].

The finding of active osteoblasts and osteoid surrounded by fibrotic tissue and hemosiderin depositions suggest that *de novo* bone formation is related to the ESW-induced intramedullary damage and induces a process of intramembranous bone formation. Interestingly, the histological images show similarities with histological images of intramembranous bone regeneration in bone marrow ablation models[25]. These models are used to examine intramembranous bone formation and implant fixation[25, 26]. In rats, the bone marrow in the femur is mechanically removed, after which regeneration occurs; such regeneration is defined by three distinct stages: an inflammatory response with clot formation; a phase of cell migration, including invasion of mesenchymal stem cells; and finally a remodeling phase to re-establish the fat and hematopoietic bone marrow. Since UESW treatment induced intramedullary damage with subsequent intramembranous bone formation, it is possible that the same biological processes are involved as in bone marrow ablation.

Our studies indicate that specific mechanisms play a role at different anatomical sites. There is subperiosteal bone formation, there is increased bone formation at existing trabecular structures, and there is intramedullary *de novo* bone formation. Most likely these are three different entities, each caused by different biological mechanisms.

Subperiosteal thickening might be related to the direct effect of shock waves on periosteal cells. In the cambium layer multipotent mesenchymal stem cells are present which can differentiate to osteoblasts and chondrocytes, and the periosteum is also a potent source for a wide variety of growth factors that are important for bone regeneration[27]. An *in vitro* experiment revealed that ESW-treated human periosteal cells have increased proliferation and higher calcium deposition, especially when low-dose ESW were used[28]. Also, treatment with ESW results in proliferation of the cambium layer as early as 4 days post-treatment[29]. In our experiments using UESW, and in other studies using low-dose focused ESW, cortical thickening at the periosteal site was observed without the presence of subperiosteal bleedings or periosteal detachment; this shows that ESW can trigger periosteal bone formation without inducing gross damage, as in fractures[30]. Interestingly, gene expression patterns of this periosteal bone formation are related to intramembranous bone formation, which is also seen in absolute stable fracture healing[30].

Recently, the pivotal regulatory role of osteocytes on bone remodelling via Wnt signalling has been elucidated[31]. Further research on the role of this mechanism in UESW induced cortical thickening, including the effect of UESW on sclerostin, is very interesting. Wnt signalling is one of the primary pathways that controls bone formation and sclerostin is a negative regulator of this signalling pathway. More specific, sclerostin antagonizes the canonicial Wnt pathway by binding to low-density lipoprotein receptor related protein 5/6 (LRP5/6)[31]. This way sclerostin

prevents the initiation of an intracellular cascade that leads to Wnt-activation and subsequently osteoblastic bone formation. Sclerostin is of specific interest because this soluble protein is exclusively secreted by osteocytes and thus it locally influences bone microarchitecture. Deficiencies in the SOST-gene, which transcribes sclerostin, indeed lead to high bone mass phenotypes as seen in rare conditions as sclerosteosis and Van Buchem disease. Furthermore, it has been shown that sclerostin is a target molecule of PTH and that mechanical loading is closely related to SOST-expression and sclerostin secretion[32-34].

UESW induced cortical thickening might stimulate Wnt signaling, but might also lead to decreased inhibitory effects, e.g. decreased secretion of sclerostin. In our studies histological observation show the presence of empty lacunae in the cortex of UESW treated bones, especially at the medial side. Control animals seem to have lower numbers of these empty lacunae, although it is technically hard to objectively score this in the current sagittal samples. These findings might indicate that UESW treatment leads to osteocytic cell death and thus to disruption of the osteocytic canalicular network. It is known that apoptosis of an osteocyte stimulates local bone remodelling via chemoattractant molecules in order to keep the bone in biomechanically optimal conditions[35]. Another interesting finding described by several authors is hypermineralisation in the existence of lacunae of dead osteocytes[36]. The mechanism of this phenomenon is yet unknown. It has been suggested that osteocytic cell death results in cessation of sclerostin production, resulting in a decline in peptides that normally prevent mineralisation of the peri-lacunar region of osteocytes, leading to uncontrolled mineralisation[36]. Another explanation suggests that deterioration of the canalicular network and subsequently deterioration of the fluid flow results in local impaired bone remodelling and hypermineralisation[37]. Further research is needed to establish whether these processes and to what extent these processes are involved in UESW induced bone formation, starting with examining the magnitude and histological and anatomical appearance of osteocytic cell death in UESW treated bones.

Although the biological processes that relate to the increase of trabecular volume fraction and *de novo* bone formation might be multi-factorial and overlap, this is not yet established. It has been shown that application of ESW results in enhanced proliferation and maturation of bone-marrow osteoprogenitors [14, 38]. Also, in segmental defects ESW stimulate the recruitment and proliferation of mesenchymal stem cells and their osteogenic potential[12]. It is interesting that, in this nonunion model, fibrous tissue is transformed into osseous tissue, which is similar to the *de novo* bone in our experiments. It is possible that ESW promote fibroblasts to transdifferentiate towards osteoblasts and chondrocytes, a phenomenon described in distraction osteogenesis[39, 40]. The finding of increased expression of TGF-b1,

BMP-2, -3, -4, -7 and VEGF after ESW treatment is probably related to these bone regenerative effects[12, 41-44]. Furthermore, in patients treated with focused ESW for osteonecrosis in the hip, immunohistochemical analysis showed an increase in Wnt 3a and a decrease in Dickkopf-1, respectively a strong stimulator and inhibitor of Wnt signalling[7]. Indeed, Wnt proteins are involved in the regeneration of injured bone and regulate the differentiation of mesenchymal precursors towards chondrocytes or osteoblasts[45, 46]. In fact, because a short increase of Wnt proteins leads to osteoblastic differentiation and intramembranous bone formation, it is worthwhile to further investigate the specific effects of ESW on Wnt signalling[47].

In another translational study we examined the effect of UESW on bone mass and biomechanical properties in an osteoporosis model. Because most patients treated for osteoporosis receive anti-resorptive treatment with bisphosphonates (mostly alendronate), we examined the effect of UESW in saline-treated and in alendronate-treated animals. In both studies, anabolic effects were seen in the trabecular and cortical bone of treated tibiae. However, the effect on trabecular bone in saline-treated animals weakened shortly after treatment, while in alendronate-treated animals this newly-formed bone remained during follow-up. Furthermore, in alendronate-treated animals the effect of UESW on cortical bone was more pronounced, which led to stiffer and stronger bone compared to saline-treated rats. However, irrespective of saline or alendronate treatment, UESW treatment led to improved biomechanical properties compared to non-UESW treated controls. These results suggest that osteoporotic patients that receive bisphosphonates might achieve further reduction of the fracture risk by a single treatment with UESW at a certain skeletal area.

In the final study, *de novo* bone formation was again found in the bone marrow. In this experiment the result was much more pronounced than in the earlier study performed in healthy male rats. The reason for this difference is not clear, but might be related to the smaller size of the female rats compared with the male rats in the first experiment. Another important difference is that, in the second study, estrogen deficiency was induced which has a strong effect on bone marrow and might influence UESW-induced effects at this site; however, this idea is speculative. Although it is difficult to establish whether the amount of intramedullary damage was also more pronounced and could be related to the large areas of *de novo* bone formation, we did observe cortical fractures which were not previously observed. These fractures were longitudinal cracks running in one of the cortical columns. Gross osseous damage with fractures has been demonstrated in focused shock wave therapy at high dosage, but not at this EFD, and certainly not with unfocused shock waves[20, 22, 23]. Because only the cortex was affected, we assume that cavitation caused these fractures[21, 48]. These fractures were not observed in our previous studies

suggesting that, in these small animals, treatment with 1000 UESW with an EFD of 0.3 mJ/mm^2 is the upper limit before gross complications occur. However, as these fractures were observed only sporadically we believe that when UESW treatment is applied in a clinical setting, or in a larger animal model, these complications will not occur. As shown in all our experiments with UESW, osseous damage is not necessary to induce anabolic effects in bone.

Our animal study provides sufficient evidence to warrant examining the effects of UESW in a clinical trial. Using dual-emission X-ray absorpiometry (DXA) the effects on bone mass can be effectively followed over time. Skeletal sites, such as the distal radius and proximal femur, are particularly interesting to examine at this stage.

We believe that UESW has the potential to be beneficial in the treatment of osteoporosis. In addition, because UESW showed pronounced new bone formation in healthy bone it might be useful in any condition in which low bone stock is the primary problem. First of all, it might be beneficial in fracture fixation of osteoporotic fractures. Here, cortical and cancellous bone loss hamper good screw fixation, which can be augmented by UESW treatment. This can be combined with standard fixation techniques in which application can be applied during the same surgical session while under anesthesia. Furthermore, it might be beneficial in revision arthroplasty, where peri-prosthetic bone loss hampers adequate fixation of new implants. Finally, it is important to examine whether the use of unfocused shock waves might be more effective in treating nonunions than the use of focused shock waves, because a biological stimulant to the surrounding of the nonunion might be more beneficial than specifically treating the site of the nonunion. Translational research is required to examine these potential applications and, when positive findings support its use, to introduce them in the clinic.

Furthermore, for clinical application, the long-term effect on bone mass of UESW treatment for osteoporosis needs to be determined. Our osteoporosis rat model shows that the magnitude of the response is high and that resorption of the newly formed bone can be prevented with alendronate; however, for clinical application the magnitude and duration of these effects still need further study. If a significant amount of new bone formation is indeed induced, and is not subsequently resorbed, then UESW may well be added to the currently available choice of treatments for osteoporosis in our clinics. Then, the ultimate question will be whether UESW therapy also results in a decrease in the incidence of osteoporotic fractures.

PULSED ELECTROMAGNETIC FIELDS

Extensive research has focused on the role of electromagnetic fields in bone metabolism. The effects have been examined in numerous different cell types using many outcome variables and many different electromagnetic fields. *In vitro* studies have shown stimulating effects in many different cell types, including rat, murine and human mesenchymal stem cells, several osteocyte-like and osteoblast-like cell lines, chondrocytes, fibroblasts and osteoclasts [42-56]. Of the many outcome variables described, an increase in growth factors of the BMP superfamily might be the most interesting finding for the clinic. In several models it was shown that pulsed electromagnetic field (PEMF) therapy stimulated the production or expression of TGF-beta 1, BMP-2, 4, 5, 6 and 7[49-53].

These *in vitro* findings warranted the performance of translational animal studies to examine the effects of PEMF on bone in a more clinically-relevant setting. Many animal models have been used, including rats, mice, turkeys, rabbits, horses and dogs[59-70]. However, the wide variety in these models, and the difference in the characteristics of the PEMF signals, makes the interpretation, comparison and clinical translation of these studies rather complicated. A few studies have been performed in a clinically-relevant setting and include a fracture model, a distraction model, and a(n) (disuse) osteoporosis model[59-63, 66, 71-76]. However, because the findings from the various models were often inconsistent, it is difficult to interpret the results in a meaningful way. For instance, in an osteotomy model in the dog, new bone formation was found at the late stage of distraction, whereas in a rabbit model this was found only in the early stage of distraction[54, 55]. One study described less bone formation in 3-week-old PEMF-treated mice compared to non-treated controls, indicating an inhibiting effect on endochondral bone formation[56], which is in contrast with *in vitro* findings that demonstrated stimulation of endochondral ossification[57].

Also in the clinical setting, controversy exists about the effects of PEMF. There is a lack of well-designed, prospective double-blind placebo-controlled studies examining the bone regenerative capacities of PEMF and other electromagnetic stimuli[58, 59]. Although observational studies suggest a stimulatory effect of PEMF on bone formation in nonunions, conclusive evidence is still lacking[58]. One of the few placebo-controlled randomized trials showed that the rate of unions between treated and sham-treated nonunions did not differ, because of the high rate of union in the placebo group[60]. Only two other placebo-controlled studies have examined the effect of electromagnetic stimulation in nonunions[61, 62]. In the former study[62], concerns about the influence of suppliers and distributors on the outcome of the study could not be refuted by the author[63]. In the second study[61], concerns were

raised about a possible bias in the study design leading to significant differences in a group of only 21 patients[64]. Thus, albeit some studies have described a clear and positive effect of PEMF, doubts remain about the validity of these studies.

The few translational studies on the role of PEMF in the treatment of osteoporosis all showed clear beneficial effects[65-67]. Also, a clinical study reported that PEMF treatment of the distal radius leads to an increase in bone mass density[68]. However, these few reports on the positive effect of PEMF on bone mass have not been corroborated by others. Indeed, if such studies had been performed but with a negative result, they might not have been published since researchers generally have a publication bias against negative results[69, 70]. Also, it is possible that PEMF works under specific circumstances, but that its effects are highly sensitive to a specific setup; this would be a serious barrier to its clinical use. However, based on the data currently available, we see no evidence that PEMF treatment has a beneficial effect on bone mass to help osteoporotic patients.

In our studies we searched for a PEMF signal that could affect bone architecture. At the start of this work we assumed that PEMF would have a positive effect on nonunions. Therefore, we decided to combine a fibula-osteotomy nonunion model with an osteoporosis model in the same rat. This would provide the advantage that each experiment had a built-in positive control. Although the fibula-osteotomy model appeared to be effective in the hands of others, in our experience it was an unreliable model with a variable outcome. In retrospect, it would have been better to first confirm the nonunion model in an independent experiment, or to investigate the effects of PEMF in the osteoporosis model independently from the nonunion model.

However, the question arises why do we not study PEMF in humans, instead of in new and complicated animal models. It should be emphasized that the PEMF protocols depend on the many (subtle) variations in PEMF parameters, that probably result in different effects in the small animal experiments relative to humans. Thus, after finding positive effects in animals, these parameters probably need to be fine-tuned for use in humans. After all, it is osteoporosis in humans that we want to treat, and PEMF is a safe, non-invasive treatment that does not cause pain or other undesired side-effects. The designated method to determine the value of PEMF in the clinical treatment of osteoporosis is with DXA assessment.

WHOLE BODY VIBRATION

Because mechanical loading is strongly related to bone mass and bone microarchitecture, many studies have focused on the interaction between these two

parameters. Although the ultimate goal is to stimulate bone formation in clinical conditions such as osteopenia and osteoporosis, most experimental work used non-osteoporotic animal models. Mechanical loading in the form of vertical vibrations would be an ideal treatment for osteoporosis: it is easy to apply and not costly. An apparent breakthrough in this area was the work of Rubin *et al.*, showing that vertical vibrations at 20 Hz and 0.3 *g* (with *g* a measure of acceleration, where 1.0 g = 9.8 m/s^2 stands for normal gravity) for 20 min per day led to anabolic effects on cancellous bone in ovariectomized sheep[71-73].

Follow-up experiments using rodents led to contradictory results. In 8-week-old mice, vibrations at 45 Hz, 0.3 *g*, 15 min/day led to less osteoclastic activity and increased bone formation at the endocortical surface at 3 weeks, and increased trabecular bone volume and cortical area at 6 weeks[74, 75]. When this same stimulus was applied in rats, no effect was found on bone formation rate[76]. In another study in which 7 and 22-month-old mice were subjected to 90 Hz with 0.3 or 1.0 *g* whole body vibrations (WBV), no effects on dynamic bone indices or on microCT assessed bone microarchitecture were found[77]. This negative effect was also shown in a study on 8-month-old ovariectomized Wistar rats, in which bone changes were analyzed using *in vivo* microCT scanning during 6-week follow-up[78]. However, in another study on ovariectomized rats, 90 Hz at 0.3 *g* WBV with an amplitude of 0.5 mm induced improved dynamic bone indices, morphometry and biomechanical properties[79]. Also, in two earlier studies it was shown that WBV at 45 Hz at 3.0 *g*, or 50 Hz at 2.0 *g*, also led to periosteal bone formation or increased BMD at the distal femur and proximal tibia, respectively, in ovariectomized rats[80, 81]. In these studies it was also shown that WBV induced an improvement in biomechanical properties.

We designed our study on WBV in a pilot and follow-up fashion: i.e. an explorative phase in which many settings were tested in a few animals was followed by a study in which the most promising settings were examined in detail with a larger number of animals. In the explorative phase, 9 different settings were tested, of which one seemed to be effective. Closer examination revealed that only one rat was responsive to WBV, also only at 3 weeks follow-up. In the follow-up experiment no rat responded to the biophysical stimuli. In future experiments, instead of evaluating the (long-term) effects on the bone itself, it may be better to measure the instant gene expression of bone anabolic genes (such as osteocalcin) using bioluminescence[82]. In this way the direct effects of mechanical vibration can be analyzed shortly after treatment, instead of analyzing the late effects on microarchitecture. In the case that osteocalcin expression is affected by a specific vibration, then *in vivo* microCT scanning is of course the designated modality to further examine this effect.

On the other hand, there is no reason *not* to perform studies with WBV in patients with osteoporosis. Indeed, some clinical trials have been performed, albeit with con-

tradicting results. In a placebo-controlled trial in which 28 subjects were subjected to 30 Hz low-magnitude vibrations for 20 min a day and 28 subjects were put on placebo machines, no effect on BMD at the hip, spine or distal radius was found[83]. In another study, three groups were analyzed (n=70), one group that received WBV three times a week, another group that did leg resistance training, and a control group[84]. At 24-week follow-up there was an increase in BMD at the femur, but total BMD and spinal BMD were not affected by WBV. Interestingly, WBV did result in increased muscle strength, as in the training group. In a study with 56 men and women, WBV did not result in micro-architectural changes measured by pQCT, but subjects who underwent WBV were able to jump higher[85]. In another study 151 post-menopausal women were allocated to do exercises for twice a week, or to do the same exercises partly in combination with WBV, or to do no exercise and receive no WBV[86]. In both exercise groups there was an increase in BMD at the spine, but not at the hip. However, the fall incidence was also reduced in both exercise groups compared to controls. Whether WBV might stimulate physical performances in post-menopausal women in order to prevent falls remains to be determined.

Moreover, WBV is interesting because it might be effective for weight reduction. In our experiments we observed that WBV considerably reduced ovariectomy-induced weight gain. Other studies also report the inhibiting effect of low-magnitude WBV on adipogenesis and body fat accumulation[87-89]. It seems that WBV affects the differentiation of adipocytes; however, more studies are needed to further elucidate the weight loss effects[90].

CONCLUSION

In conclusion, osteoporosis is a comprehensive disease mainly affecting the middle-aged and elderly. Osteoporotic fractures lead to a loss in quality of life and increased mortality. In the future, the high incidence of osteoporosis is expected to rise even further, and prevention of all osteoporotic fractures remains the primary goal of treatment. Therefore, identification of patients with low bone mass, the treatment of these patients, and fall prevention are the key elements for reduction of fracture risk in osteoporosis.

The work in this thesis examined potential novel treatments for osteoporosis. It was shown that local treatment with UESW induces favorable effects on bone microarchitecture and its related mechanical properties. Therefore, UESW is a potential therapy to stimulate bone mass locally in order to prevent fractures; however, the effects of this therapy need to be further elucidated and confirmed in both translational and clinical studies.

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09
Summary

Osteoporosis is a disease characterized by diminished bone mass and deterioration of the bone microarchitecture leading to a higher susceptibility for fractures. Osteoporotic fractures lead to a loss in quality of life and increased mortality, especially in elderly patients. In the search for a therapy for osteoporosis other than the currently applied pharmaceutical treatment, we examined the effects of several biophysical stimuli on bone microarchitecture in small animal models.

Mechanical loading is strongly related to bone mass and therefore whole body vibrations (WBV) might serve as treatment in osteoporosis. In **Chapter 2** osteoporotic rats were put on vibrating plates. To simulate osteoporosis female rats were ovariectomized. Multiple treatment groups were examined in which the characteristics of the vibrations varied in frequency (2 or 45 Hz), acceleration (0.5 or 2.0*g*) and number of treatments. Bone changes in the proximal tibia were analyzed with the use of *in vivo* microCT during 10 week follow-up. Preventive effects on trabecular bone loss were found in a pilot study, in which 2 rats per condition were examined. However, when this same condition was further examined in larger treatment groups no effect of WBV on bone microarchitecture was found. Furthermore, we did show that WBV reduced ovariectomy induced weight gain. An effect of WBV on weight has also been shown by others and demonstrates that WBV might play a role in weight management. We found no support for the use of WBV in the treatment of osteoporosis.

The second biophysical stimulus that we examined is Pulsed electromagnetic fields (PEMF). These have been shown to induce cellular effects that affect bone adaptation. It is used in the treatment of nonunions. In **Chapter 3 and 4** we examined if PEMF could affect bone microarchitecture in healthy and ovariectomized rats. First, ovariectomized rats were exposed to a systemic treatment with PEMF. Four different treatment protocols based on previous experimental studies and based on clinically used PEMF signals were studied. Bone changes were analyzed with the use of microCT scanning. We could not demonstrate an effect of PEMF on bone microarchitecture during 6 week follow-up.

In another study we examined the effect of locally applied PEMF in healthy and ovariectomized rats. The electromagnetic fields in this study were stronger than the fields used in the study with systemic PEMF treatment. To also examine the effect on fracture healing a fibular osteotomy was created. No effect of PEMF could be demonstrated on bone microarchitecture nor on fracture healing. The results of our studies do not substantiate a potential role for the use of PEMF in the treatment of osteoporosis.

The final biophysical stimulus that was studied in this thesis is unfocused extracorporeal shock waves (UESW). In **Chapter 5, 6 and 7** we explored the potential role of UESW in the treatment of osteoporosis. In **Chapter 5** the effect of unfocused

shock waves on the bone microarchitecture in ovariectomized rats was examined. Again *in vivo* microCT scanning was used to analyze cancellous and cortical bone changes. The hind leg of ovariectomized and non-ovariectomized rats were treated with 2000 UESW with an energy flux density (EFD) of 0.16 mJ/mm^2. Treatment consisted of one time 2000 shock waves or twice 1000 shock waves with an interval of 3 weeks. Furthermore, rats were treated 3 weeks after ovariectomy, when bone loss was fair, or 10 weeks after ovariectomy when bone loss was severe. Treatment resulted in an increased trabecular volume fraction of 10%, higher connectivity density and more plate like trabeculae in sham-ovariectomized rats at 7 week follow-up. In ovariectomized rats with fair bone loss the effect was much smaller. Only when treated with 2000 UESW 3 weeks after ovariectomy there was a significant higher trabecular volume fraction at 7 weeks after treatment. On cortical bone there was no effect and no *de novo* bone formation was observed. In ovariectomized rats with severe bone loss UESW was not effective. In this study it was shown that UESW primarily affected existing trabecular structures.

In a follow-up experiment we examined the effect of UESW on bone turnover with the use of multi-pinhole SPECT-scanning. When no effect on bone turnover was found when UESW with an EFD of 0.16 mJ/mm^2 were used, UESW with an EFD of 0.3 mJ/mm^2 were examined. With these higher energy UESW large effects on bone turnover were demonstrated. These results are presented in **Chapter 6**. Furthermore, it was shown with *in vivo* microCT scanning that trabecular volume fraction and cortical volume increased and that UESW treated bones had a higher stiffness with biomechanical testing. Histological examination and reconstructed microCT scans showed areas of *de novo* bone formation in the bone marrow. Active intramembranous bone formation was observed. Although the exact biological mechanism of these bone forming areas is not known it is likely related to an overall damage to the bone marrow, which we observed after UESW treatment. No osseous damage or periosteal damage was found.

In **Chapter 7** we examined if UESW can also affect bone microarchitecture and biomechanical properties in ovariectomized rats. The hind leg of ovariectomized rats were treated with 1000 UESW with an EFD of 0.3 mJ/mm^2. Because most patients with osteoporosis receive bisphosphonates we performed UESW treatment in ovariectomized rats that did or did not receive an anti-resorptive treatment with the bisphosphonate Alendronate. UESW induced a pronounced increase in trabecular bone volume shortly after treatment in both saline and Alendronate treated animals. However, in Alendronate treated animals these effects remained during 10 week follow-up in contrast to saline treated rats in which the effects diminished at later follow-up. In both saline and Alendronate treated rats UESW induced periosteal bone formation and resulted in improved biomechanical properties. On histology

huge areas of intramembranous *de novo* bone formation were again found in the bone marrow. We hypothesize that various biological mechanisms are responsible for the effects of UESW on cancellous, cortical and *de novo* bone formation.

In conclusion, we demonstrated that UESW treatment is a potential therapy to stimulate bone mass locally in order to prevent osteoporotic fractures; however, the effects of this therapy need to be further elucidated and confirmed in translational and clinical studies. We found no support for the use of WBV or PEMF in the treatment of osteoporosis.

10 Appendices

Nederlandse samenvatting
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NEDERLANDSE SAMENVATTING

Osteoporose wordt gekenmerkt door een verlies van bot en een achteruitgang van de bot-microarchitectuur. Hierdoor is de kans op het krijgen van botbreuken verhoogd. Osteoporotische botbreuken leiden tot een verhoogde mortaliteit en morbiditeit, met name bij ouderen. Op zoek naar een alternatieve behandeling voor de huidige medicamenteuze behandeling, hebben wij de effecten van verschillende biofysische stimuli op bot-microarchitectuur onderzocht in verschillende proefdiermodellen.

Mechanische belasting en botmassa zijn nauw met elkaar verbonden. Mechanische trillingen zouden daarom een behandeling voor osteoporose kunnen zijn. In **hoofdstuk 2** worden de effecten van 'Whole Body Vibrations' (WBV) beschreven. Om osteoporose te simuleren wordt bij vrouwelijke ratten een bilaterale ovariëctomie uitgevoerd. Verschillende groepen werden onderzocht, waarbij frequentie (2 of 45 Hz), versnelling (0,5 of 2,0*g*) en het aantal behandelingen werden gevarieerd. Met *in vivo* microCT werden botveranderingen in de proximale tibia gedurende een periode van 10 weken geanalyseerd. In een pilotstudie, waarbij we twee ratten per conditie analyseerden, vonden we in één groep preventieve effecten op het botverlies. Echter, toen we in een vervolgexperiment dezelfde trilling op een grotere groep dieren onderzochten, konden we geen effect op de bot-microarchitectuur meer aantonen. Wat we wel vonden was dat geovariëctomeerde ratten minder in gewicht toenamen dan ratten die niet getrild werden. Effecten van WBV op gewicht is al eerder beschreven en geeft aan dat WBV een rol kan spelen in gewichtsreductie. We hebben geen aanwijzingen dat WBV een rol kan spelen bij de behandeling van osteoporose.

De tweede biofysische stimulus die we onderzocht hebben is Pulserende Elektromagnetische Velden (PEMV). Het is aangetoond dat PEMV cellulaire effecten hebben die botadaptatie beïnvloeden. Het wordt gebruikt in de behandeling van pseudartrosen. In de **hoofdstukken 3 en 4** hebben we onderzocht of PEMV de bot-microarchitectuur in gezonde of osteoporotische ratten kan beïnvloeden. Eerst werd een systemische behandeling met PEMV bij geovariëctomeerde ratten onderzocht. Vier verschillende PEMV-signalen die gebaseerd waren op eerdere experimentele studies en op klinisch toegepaste PEMV-signalen werden onderzocht. Veranderingen in het bot werden geanalyseerd met microCT. Gedurende 6 weken werden de dieren gevolgd, maar we konden tijdens die periode geen effecten zien van PEMV op de bot-microarchitectuur. In een tweede studie hebben we het effect van lokaal toegepaste PEMV onderzocht in zowel gezonde als osteoporotische ratten. In deze vervolgstudie was het elektromagnetisch veld sterker dan bij de eerste studie met een systemische behandeling en hebben we ook het effect op

botgenezing onderzocht door een fibula-osteotomie in het model op te nemen. We konden geen effect van PEMV aantonen, niet op de bot-microarchitectuur en ook niet op fractuurgenezing. Beide studies leveren geen aanwijzingen op dat PEMV eventueel een rol kan spelen in de behandeling van osteoporose.

De laatste biofysische stimulus die in dit proefschrift onderzocht werd. is ongefocusseerde extracorporale schokgolftherapie, ofwel 'Unfocused Extracorporeal Shock Wave therapy'. **In hoofdstukken 5, 6 en 7** hebben we de mogelijke rol van 'Unfocused Extrcorporeal Shock Waves' (UESW) voor de behandeling van osteoporose onderzocht. In **hoofdstuk 5** werd het effect van UESW op de bot-microarchitectuur in geovariëctomeerde ratten onderzocht. Opnieuw werd *in vivo* microCT scanning gebruikt om de veranderingen in het trabeculaire en het corticale bot te volgen. De achterpoten van geovariëctomeerde en gezonde ratten werden behandeld met 2000 schokgolven met een energieniveau, ofwel 'energy flux density' (EFD), van 0,16 mJ/mm^2. De behandeling bestond uit eenmalig 2000 schokgolven of tweemaal 1000 schokgolven met een tussenperiode van 3 weken. Daarnaast werden er ratten behandeld 3 weken na ovariëctomie, wanneer er mild botverlies was opgetreden, en 10 weken na ovariëctomie, wanneer er uitgesproken botverlies was opgetreden. In gezonde, niet-geovariëctomeerde ratten zorgde de behandeling na 7 weken voor 10% meer trabeculair bot, een hogere connectiviteit van de trabekels, waarbij de trabekels hun plaatstructuur meer hadden behouden. In geovariëctomeerde ratten met mild botverlies waren de effecten veel kleiner. Alleen wanneer er 2000 schokgolven eenmalig werden gegeven was er na 7 weken significant meer trabeculair bot. Op corticaal bot zagen we geen effect. In geovariëctomeerde ratten met ernstig botverlies konden we geen effect van UESW op het bot aantonen. Deze studie toonde aan dat UESW met name de bestaande structuren lijkt te beïnvloeden.

In een vervolgexperiment hebben we het effect van UESW op de botremodelering onderzocht middels multi-pinhole SPECT-scanning. Toen we geen effecten op de botremodelering zagen als we UESW met een EFD van 0,16mJ/mm^2 gaven, hebben we vervolgens het effect van UESW met een EFD van 0,3 mJ/mm^2 onderzocht. Met dit hogere energieniveau zagen we grote effecten op botremodelering. Deze resultaten staan vermeld in **hoofdstuk 6**. We hebben in die studie ook aangetoond dat bij dit hogere energieniveau er uitgesproken meer trabeculair en corticaal bot ontstaat. Bij biomechanische testen blijken de botten ook stijver en sterker te zijn. Bij histologisch onderzoek en ook op gereconstrueerde microCT scans konden in het beenmerg gebieden met nieuwe botstructuren aangetoond worden. Actieve intramembraneuze botvorming was daar aanwezig. Het biologische principe achter deze botnieuwvorming is nog niet opgehelderd, maar de schade die we in het

beenmerg na een behandeling zagen, houdt hier waarschijnlijk verband mee. In het bot en het periost hebben we geen schade aan kunnen tonen.

In **hoofdstuk 7** hebben we onderzocht of UESW met een EFD van 0,3 mJ/mm^2 de bot-microarchitectuur en biomechanische eigenschappen ook in geovariëctomeerde ratten kan beïnvloeden. Omdat in de klinische praktijk patiënten met osteoporose behandeld worden met een resorptieremmer, hebben we in deze studie geovariëctomeerde ratten met en zonder de resorptieremmer alendroninezuur (o.a. Fosamax) onderzocht. In beide groepen leidde UESW tot een sterke verhoging van de hoeveelheid trabeculair bot, echter in de groep die ook alendroninezuur kreeg bleef dit effect gedurende de vervolgperiode van 10 weken bestaan, terwijl in de groep die deze resorptieremmer niet kreeg de effecten van voorbijgaande aard waren. In beide groepen was er een sterk verhoogde corticale botaanmaak aan de periostale zijde. Deze effecten resulteerden ook in verbeterde biomechanische eigenschappen. Met histologie konden wederom grote gebieden in het beenmerg aangetoond worden waar intramembraneuze botnieuwvorming optreedt. Wij denken dat verschillende biologische principes een rol spelen bij de effecten van UESW op het trabeculaire bot, het corticale bot en de botnieuwvorming in het beenmerg.

Concluderend hebben we aangetoond dat UESW de lokale botmassa sterk kan doen toenemen, hetgeen osteoporotische fracturen in potentie zou kunnen voorkomen. Echter dienen de biologische effecten van deze nieuwe toepassing verder uitgezocht te worden en zijn er klinische vervolgstudies nodig om het positieve effect te bevestigen. We hebben geen potentiële toepassing van WBV of PEMV voor de behandeling van osteoporose aan kunnen tonen.

DANKWOORD

Tot slot wil ik eenieder bedanken die een bijdrage heeft gehad in de totstandkoming van dit proefschrift. Een aantal mensen zou ik nog in het bijzonder willen bedanken:

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CURRICULUM VITAE

Olav Pieter van der Jagt werd geboren op 18 februari 1980 te Groningen. Hij groeide op in het pittoreske en rustige Eelde-Paterswolde alwaar hij naar basisschool 'De Ekkel' ging. Het VWO volgde hij vervolgens op het Maartens college, eerst in Groningen en later in Haren. In 1998 begon hij aan zijn studie geneeskunde aan de Rijksuniversiteit Groningen. In Berlijn bij de afdeling experimentele chirurgie van het Charité ziekenhuis werd voor het eerst zijn belangstelling voor translationeel onderzoek gewekt. Hij deed daar onderzoek naar kunstlevers onder begeleiding van Dr. Igor Sauer. Terug in Nederland liep hij zijn co-schappen in het Scheper ziekenhuis in Emmen. Het was hier waar zijn belangstelling voor orthopedie gewekt werd. Toen hij na het behalen van zijn doctoraal en artsdiploma de kans kreeg om op het orthopedisch onderzoekslaboratorium van het Erasmus MC translationeel onderzoek te kunnen doen, greep hij die dan ook met beide handen aan. Onder begeleiding van onder andere zijn promotoren prof. H. Weinans en prof. J.A.N. Verhaar heeft hij daar bijna vier jaar lang experimenteel botonderzoek mogen doen. In 2009 begon zijn vooropleiding chrirurgie in het Sint Franciscus Gashuis, Rotterdam (opleider dr. A.J.H. Kerver). In 2011 startte hij met zijn opleiding tot orthopaedisch chirurg. Na een jaar in het Erasmus MC gewerkt te hebben (opleider prof. J.A.N. Verhaar), werkt hij momenteel in het Elisabeth ziekenhuis in Tilburg (opleider dr. J. de Waal Malefijt). Hier zal hij de komende twee jaar zijn opleiding volgen om vervolgens weer terug te keren in het Erasmus MC om de opleiding af te ronden. Samen met Nicole Kops heeft hij een fantastische zoon. De tweede spruit is op komst en wordt in december 2012 verwacht.

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Van der Jagt OP, Van der Linden JC, Waarsing JH, Verhaar JA, Weinans H. Systemic treatment with pulsed electromagnetic fields do not affect bone microarchitecture in osteoporotic rats. *International Orthopedics* 2012; 36(7): 1501-6

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Van der Jagt OP, Van der Linden JC, Waarsing JH, Verhaar JA, Weinans H. Pulsed Electromagnetic Fields do not affect bone microarchitecture in osteoporotic or healthy rats. *Submitted*

Van der Stok J, Van der Jagt OP, Amin Yavari S, De Haas MFP, Waarsing JH, Jahr H, Van Lieshout EMM, Patka P, Verhaar JAN, Zadpoor AA, Weinans H. Selective laser melting produced porous titanium scaffolds regenerate bone in critical size cortical bone defects. *Submitted*

Piscaer TM, De Jong M, Van der Jagt OP, Botter SM, Verhaar JAN, Weinans H. Real-time assessment of bone metabolism in small animal models for osteoarthritis using MPH-SPECT/CT. *Submitted*

PHD PORTFOLIO

PHD TRAINING

General courses	**Year**	**Workload (ECTS)**
Animal science course, artikel 9	2005	4.0 ECTS
Classical Methods for data-analysis	2008	4.0 ECTS
Presentations		
Various presentations at research meetings of the department of orthopedics	2005-2008	4.0 ECTS
Presentations (inter)national conferences		
ISMST Basic Research Meeting, Innsbruck 'Unfocused Extracorporeal shock waves in Osteoporosis'	2012	1.0 ECTS
Orthopaedic Trauma Association, Baltimore 'Unfocused Extracorporeal shock waves induce anabolic responses in osteoporotic bone'	2010	1.0 ECTS
European Orthopaedic Research Society, Davos 'Unfocused Extracorporeal shock waves induce anabolic responses in osteoporotic bone'	2010	1.0 ECTS
Orthopaedic Research Society, New Orleans 'Unfocused Extracorporeal shock waves as potential treatment for Osteoporosis'	2010	1.0 ECTS
ISMST Basic Research meeting, Wenen 'Unfocused Extracorporeal shock waves as potential treatment for Osteoporosis'	2010	1.0 ECTS
Nederlandse Vereniging voor Orthopaedie, Utrecht 'Unfocused Extracorporeal shock waves as potential treatment for Osteoporosis'	2010	1.0 ECTS
Nederlandse Vereniging voor Calcium- en Botstofwisseling, Zeist 'Diminished Bone Loss and Enhanced Bone Turnover After Treatment with Unfocused Shockwaves in Healthy and Osteoporotic Rats'	2008	1.0 ECTS
Bone-Tec, Hannover 'Endochondral ossification: an alternative approach for bone repair?'	2008	1.0 ECTS
Nordic Orthopaedic Federation, Amsterdam	2008	1.0 ECTS

'Diminished Bone Loss and Enhanced Bone Turnover After Treatment with Unfocused Shockwaves in Healthy and Osteoporotic Rats'		
Nederlandse Vereniging voor Calcium- en Botstofwisseling, Zeist	2007	1.0 ECTS
'Anabolic effects of PTH(1-34) on cortical and cancellous bone highly depend on mechanical feedback'		
Tissue Engineering International and Regenerative Medicine Society, Londen	2007	1.0 ECTS
'An *in vitro* endochondral ossification model using human MSCs'		

Poster presentations

AO foundation, Where science meets clinics, Davos	2011	1.0 ECTS
'Unfocused extracorporeal shock waves induce anabolic responses in osteoporotic bone'		
Osteoarthritis Research Society International, Rome	2008	1.0 ECTS
'Endochondral ossification: an alternative approach for bone repair?'		
Orthopaedic Research Society, San Francisco	2008	1.0 ECTS
'Chondrogenic differentiated pellet cultures of hBMSCs produce VEGFa and MMPs'		
Orthopaedic Research Society, San Francisco	2008	1.0 ECTS
'Unfocused extracorporeal shockwave therapy diminishes bone loss in rats'		
American Society for Bone and Mineral Research, Honolulu	2007	1.0 ECTS
'Dynamic bone changes in PTH(1-34) treated ovariectomized rats, evaluated with *in vivo* microCT scanning'		
American Society for Bone and Mineral Research, Honolulu	2007	1.0 ECTS
'Unfocused extracorporeal shockwave therapy diminishes bone loss in ovariectomized rats'		
American Society for Bone and Mineral Research, Honolulu	2007	1.0 ECTS
'Extracorporeal Shockwave Therapy Stimulates Bone remodeling in the rat, evaluated with *in vivo* micro-SPECT scanning'		

Teaching

Supervising Master's thesis C.J. Van Manen: 'Invloed van Pulserende Elektromagnetische Velden (PEMV) op endochondrale ossificatie'	2007	4.0 ECTS
Practicals in bone pathology 2nd years medical students	2007-2009	2.0 ECTS
Supervising 4th year medical students in writing review article	2008-2009	2.0 ECTS

LIST OF ABBREVIATIONS

ADL	activities of daily living
ANOVA	analysis of variance
BMD	bone mineral density
BMP	bone morphogenetic protein
BV/TV	bone volume fraction
Conn/TV	connectivity density
CtTh	cortical thickness
CtV	cortical volume
DXA	dual emission/energy X-ray absorptiometry
EFD	energy flux density
EMF	electromagnetic fields
ERK	extracellular regulated kinase
ESW	extracorporeal shock waves
ESWT	extracorporeal shock wave therapy
H&E	hematoxylin and eosin
IGF	insulin-like growth factor
MAPK	mitogen-activated protein kinase
microCT	micro-computed tomography
MPH	multi-pinhole
NSAID	non-steroidal anti-inflammatory drugs
OVX	ovariectomy
PEMF	pulsed electromagnetic fields
pQCT	peripheral quantitative computed tomography
PTH	parathyroid hormone
PTHrP	parathyroid hormone related peptide
SD	standard deviation
SMI	structure model index
SPECT	single-photon emission computed tomography
TbTh	trabecular thickness
TGF	transforming growth factor
UESW	unfocused extracorporeal shock waves
VEGF	vascular endothelial growth factor
WBV	Whole body vibrations

Biophysical stimuli as potential treatment for osteoporosis