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● *Original Contribution*

RADIAL EXTRACORPOREAL SHOCK WAVE THERAPY (RESWT) INDUCES NEW BONE FORMATION *IN VIVO*: RESULTS OF AN ANIMAL STUDY IN RABBITS

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Abstract—The aim of this study was to investigate if radial extracorporeal shock wave therapy (rESWT) induces new bone formation and to study the time course of ESWT-induced osteogenesis. A total of 4000 impulses of radial shock waves (0.16 mJ/mm^2) were applied to one hind leg of 13 New Zealand white rabbits with the contralateral side used for control. Treatment was repeated after 7 days. Fluorochrome sequence labeling of new bone formation was performed by subcutaneous injection of tetracycline, calcein green, alizarin red and calcein blue. Animals were sacrificed 2 weeks (n = 4), 4 weeks (n = 4) and 6 weeks (n = 5) after the first rESWT and bone sections were analyzed by fluorescence microscopy. Deposits of fluorochromes were classified and analyzed for significance with the Fisher exact test. rESWT significantly increased new bone formation at all time points over the 6-week study period. Intensity of ossification reached a peak after 4 weeks and declined at the end of the study. New bone formation was significantly higher and persisted longer at the ventral cortex, which was located in the direction to the shock wave device, compared with the dorsal cortex, emphasizing the dose-dependent process of ESWT-induced osteogenesis. No traumata, such as hemorrhage, periosteal detachment or microfractures, were observed by histologic and radiologic assessment. This is the first study demonstrating low-energy radial shock waves to induce new bone formation *in vivo*. Based on our results, repetition of ESWT in 6-week intervals can be recommended. Application to bone regions at increased fracture risk (*e.g.*, in osteoporosis) are possible clinical indications.
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Key Words: **Lithotripsy, Shockwave, Osteogenesis, Bone growth, ESWL.**

INTRODUCTION

Extracorporeal shock wave therapy (ESWT) has been introduced to treat a variety of soft tissue pathologies and high-quality randomized trials demonstrated effectiveness especially for enthesiopathies like plantar fasciitis or calcific tendonitis of the shoulder (Gerdesmeyer et al. 2003, 2008; Gollwitzer et al. 2007; Diehl et al. 2011). Furthermore, multiple studies indicated that high-energy focused ESWT might also be appropriate to stimulate bone healing in delayed unions and nonunions (Elster et al. 2010; Alvarez et al. 2011). Recently, activation of bone regeneration in a vascular bone necrosis (Wang CJ et al. 2005) and stimulation of fracture healing have been reported (Moretti et al. 2009; Wang CJ et al. 2006).

Focused shock waves have demonstrated to induce new bone formation in various animal models, both on normal, fractured and osteomized bone and bone defects (Delius et al. 1998; Chen et al. 2004a; van der Jagt et al. 2011). Disclosed mechanisms include the induction of oxygen radicals and membrane hyperpolarization, followed by the expression of growth factors and stimulation of osteoprogenitor cells (Wang FS et al. 2001, 2002a, 2003; Chen et al. 2004a). To activate bone healing in the clinical setting, ESWT is commonly performed with high-energy shock waves requiring some kind of anesthesia and repeated interventions in intervals of 4–6 weeks (Elster et al. 2010; Alvarez et al. 2011). However, there are neither data available on the minimum energy required for bone stimulation, nor data on the dynamic and persistence of ESWT-induced osteogenesis. Current

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treatment recommendations are mainly based on empirical rather than controlled experimental data.

Radial ESWT (rESWT) is a relatively new and cost-effective method of shock wave application. Radial shock waves are generated ballistically by accelerating a bullet to hit an applicator, which finally transforms the kinetic energy into radially expanding pressure waves (Gerdesmeyer et al. 2004). Compared with the commonly used focused shock waves, rESWT is characterized by a larger treatment area, which simplifies application by reflecting pathology zone rather than a point (Gerdesmeyer et al. 2004). Furthermore, radial shock waves miss the typical steepening of focused shockwaves and, therefore, are physically more correctly classified as pressure waves. rESWT is considered critically with bone pathologies because of to its unfocused distribution and lower energy level, both resulting in reduced tissue penetration.

The present study was conducted to investigate the effect of rESWT on bone formation and to study the time course of ESWT-induced osteogenesis, which is mandatory to establish the most effective treatment protocol for bone stimulation.

METHODS

Shock wave treatment

The present study was approved by the animal use and care committee of the regional government (Regierung von Oberbayern). A total of 13 female New Zealand white rabbits (3.5–4.5 kg) were included in the animal model. Radial shock waves were applied with a Swiss Dolorclast shock wave device (EMS Electro Medical Systems, Nyon, Switzerland) to one randomized femur of each animal, while the contralateral side served as intraindividual control. Prior to each treatment, the animals were anesthetized with medetomidine, ketamine and metamizole, and the left hind-leg was shaved. The application site was localized at the ventral thigh, precisely superior to the patella with the rabbit in supine position and the knee joint in 45 degree flexion. rESWT was applied with an ultrasound transmission gel used as contact medium with the following parameters: impulse count 4000 per intervention, impulse rate 8/s, pressure 4 bar, and energy flux density 0.16 mJ/mm^2. The treatment was repeated with similar preparation 7 days after the first intervention. A flowchart of the study protocol is provided in Table 1.

Polychrome sequence labeling of newly formed bone

To allow microscopic work-up of new bone formation, polychrome sequence labeling was performed with different clearly contrasting fluorescent dyes administered subcutaneously once per day. Intravital staining with tetracycline was started prior to treatment to label the baseline value, followed by injection of calcein green, alizarin red and calcein blue after completion of both shock wave sessions (Table 1).

Table 1. Treatment interventions, fluorochrome application and end points

Day	Intervention	Sacrifice
−4 to −1	tetracycline (25 mg/kg s.c.)	
0	rESWT (4000 impulses, 0.16 mJ/mm^2, 4 bar, 8 Hz)	
7	rESWT (4000 impulses, 0.16 mJ/mm^2, 4 bar, 8 Hz)	
11–13	calcein green (20 mg/kg s.c.)	
14		Group I (n = 4)
25–27	alizarin red (30 mg/kg s.c.)	
28		Group II (n = 4)
38–41	calcein blue (30 mg/kg, s.c).	
42		Group III (n = 5)

rESWT = radial extracorporeal shock wave therapy.

Analysis of new bone formation

Animals were sacrificed at 2 weeks (n = 4), 4 weeks (n = 4) and 6 weeks (n = 5) after the first rESWT (Table 1) with an overdose of pentobarbital. Rabbit femurs with adjacent soft tissues were removed carefully and contact radiographs were taken. Fixation was carried out in 100% (v/v) methanol for one week, followed by dehydration in ethanol 100% (v/v) for 5 days, and defatting in xylol for 24 h. Bone samples were embedded in PMMA. Thereafter, sagittal sections with a thickness of approximately 75 μm were cut and investigated with broad-band fluorescence microscopy. Visualization of tetracycline, calcein green and alizarin red was achieved with Filter 09 (Carl Zeiss MicroImaging GmbH, Jena, Germany). Filter 02 (Carl Zeiss MicroImaging GmbH) was used to investigate alizarin red and calcein blue bands of new bone formation. The fluorescing bands were analyzed, and type of fluorochrome, intensity, extension and localization (endosteal/periosteal; ventral/dorsal cortex) were documented.

The magnitude and distribution of newly formed bone was evaluated by blinded review according to the classification provided in Table 2. The total accumulated ossification bands (independent of the type of fluorochrome) were classified with rating system A, which was modified after Maier et al. (Maier et al. 2004). For the assessment of osteogenetic activity at the different time points (analyses of the single fluorochrome bands), the rating system was modified to a total of five different intensities (rating system B). Microscopic work-up further included a qualitative histologic analysis for microtraumata such as fractures, hematomas and periosteal detachment.

Table 2. Classification of new bone formation

Rating system A: total accumulated new bone formation	
Intensity of new bone formation	Class
No signs of new bone formation	0
Sporadic new endosteal and/or periosteal bone formation, without covering the entire bone surface	1
New endosteal and/or periosteal bone formation, covering the entire bone surface	2
Rating system B: new bone formation at specific time points	
Intensity of new bone formation	Class
No signs of new bone formation or only weak, inhomogeneous fluorescent band	0
Homogeneous band of new bone formation at one cortex only, with low intensity/smooth borders	1
Homogeneous band of new bone formation at one cortex only, with high intensity/sharp delineation	2
Homogeneous bands of new bone formation at both cortices, with low intensity/smooth borders	3
Homogeneous bands of new bone formation at both cortices, with high intensity/sharp delineation	4

Radiologic work-up was performed with contact radiographs (3 mA, 35 kV, 60 s) before and microradiographs (3 mA, 15 kV, 45 s) after sectioning of the explanted femurs. Assessment included new periosteal and endosteal bone formation, callus formation, cortical and trabecular fractures, and periosteal detachment. The lungs of all animals were also harvested and examined both macroscopically and histologically for signs of embolism or dislocated bone trabeculae within pulmonary vessels, which had been previously described after the application of high-energy ESWT (Maier et al. 2003a).

Statistical analysis of new bone formation in treated and untreated femora was performed with the Fisher exact test, with $p < 0.05$ considered statistically significant.

RESULTS

The present study was conducted to investigate the effect of low-energy radial shock waves on osteogenesis and to study the dynamics of ESWT-induced new bone formation. Thus, radial shock waves were applied to the distal femur of New Zealand white rabbits and fluorescent sequence labeling of newly formed bone was realized with different fluorescent dyes. Integration of the fluorescent dyes into bands of newly deposited bone was shown by fluorescence microscopy and was significantly increased after rESWT (Figs. 1, 2 and 3). The different colored fluorescent dyes allowed a description of the time course of new bone formation. Sharp and homogeneous bands of integrated fluorochromes were observed in all bone specimens treated with rESWT (Fig. 1), whereas examination of the untreated contralateral femurs demonstrated only sporadically weak signs of new bone formation that persisted at this very minor grade over the entire study period (Figs. 1 and 4).

Significant induction of bone formation by rESWT could be demonstrated already in the first week after shock wave application and persisted at least until week 6, which was documented by the newly formed fluorescent bands with dyes administered in the late phase of the experiment (alizarin red and calcein blue, Figs. 2 and 4). New bone formation reached a peak after 4 weeks and declined to lesser intensity 6 weeks after shock wave application (Fig. 4). Nevertheless, osteogenesis after rESWT was significantly increased compared with the untreated control at all time points ($p < 0.05$).

Differentiation of rESWT-induced osteogenesis at the ventral and dorsal femoral cortex was carried out because shock waves were applied to the ventral thigh and a distance-related decline of shock wave energy in bone was expected. Compared with the untreated control side, osteoneogenesis was significantly increased at the ventral cortex at all time points (Fig. 5a) and at the dorsal cortex for approximately 4 weeks after rESWT (Fig. 5b). Thereafter, new bone formation declined at the dorsal cortex to values indifferent of the untreated control (Fig. 5b). When both cortices were compared, rESWT-induced bone formation reached significantly higher levels at the ventral cortex compared with the dorsal cortex in the early phase (calcein green, $p = 0.031$) and in the late phase of the experiment (calcein blue, $p < 0.008$) but not during the peak of osteogenesis at 4 weeks (alizarin red, $p = 0.206$). No significant differences were observed with regard to endosteal and periosteal bone formation ($p > 0.05$) and no significant signs of new bone formation were observed in trabecular bone.

Contact radiographs and or microradiographs were negative for calcified bone remodeling, bone resorption, osteolysis or callus formation. Furthermore, no trabecular or cortical fractures were detected. Qualitative histology did not show intraosseous bleeding, periosteal detachment or microfractures. Furthermore, neither signs of pulmonary embolisms nor displaced bone fragments were observed in the lung sections. No side effects of rESWT were found but some hematoma at the application site.

DISCUSSION

In an effort to achieve bone healing in a noninvasive way, several experimental and clinical studies investigated ESWT for bone stimulation and indicated improved bone union and increased bone turnover after the application of focused high-energy shock waves (Elster et al. 2010; Alvarez et al. 2011; van der Jagt

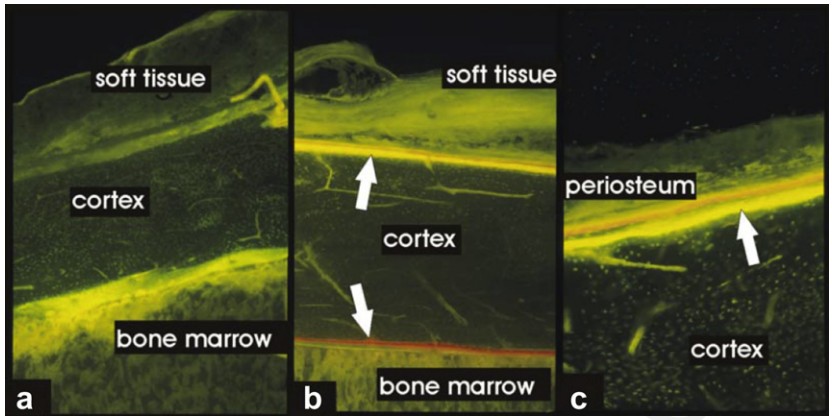

Fig. 1. Fluorochrome sequence labeling of new bone formation at the ventral cortex of rabbit femurs, investigated 4 weeks after the first radial extracorporeal shock wave therapy (rESWT) application: (a) untreated bone (magnification ×50, Zeiss Filter 09); (b) rESWT treated bone (magnification ×50) and (c) rESWT treated bone (magnification ×100). Arrows indicate bands of both periosteal and endosteal new bone formation.

et al. 2011). Whereas a positive effect of ESWT on healing of nonunions has been described in most published studies, proof of effectiveness by means of a experimental study is still lacking (Gollwitzer et al. 2006). Furthermore, recommendations on treatment parameters such as energy flux density, impulse rate, number of treatment interventions and treatment free intervals vary considerably and are mainly based on empirical data of uncontrolled trials (Gollwitzer et al. 2006). Basic research has provided a better understanding on the mechanisms of ESWT and its interaction with bone. However, data on the dynamics of ESWT-induced osteogenesis are rare in spite of the high clinical relevance to determine the most appropriate treatment protocols. Furthermore, new bone formation after the application of radial, unfocused ESWT, which might be advantageous by addressing larger treatment areas, has not been investigated so far. The present study is the first investigation on the dynamics of ESWT-induced bone formation and the osteogenetic potential of radial shock waves.

Principles of shock wave therapy

Shock waves can be generated by electrohydraulic, electromagnetic or piezoelectric methods or (like radial shock waves in the present study) by pneumatic acceleration of an applicator bullet within the hand piece (Gerdesmeyer et al. 2002, 2004). Whereas "conventional" shock waves known from lithotripsy are focused to a zone of highest energy in front of the applicator, radial shock waves are unfocused and distributed in a radial manner. Consequently, radial shock waves reach lower energy flux densities but address greater treatment areas (Gerdesmeyer et al. 2002, 2004). Shock waves are single high amplitude sound waves that propagate in tissue with a sudden rise from ambient pressure to its

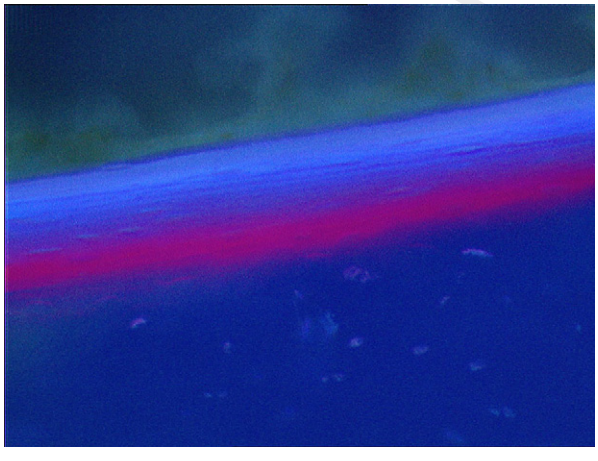

Fig. 2. Endosteal fluorochrome deposition of alizarin red and calcein blue documented persisting new bone formation 6 weeks after first radial extracorporeal shock wave therapy (rESWT) (magnification ×100, Zeiss filter 02).

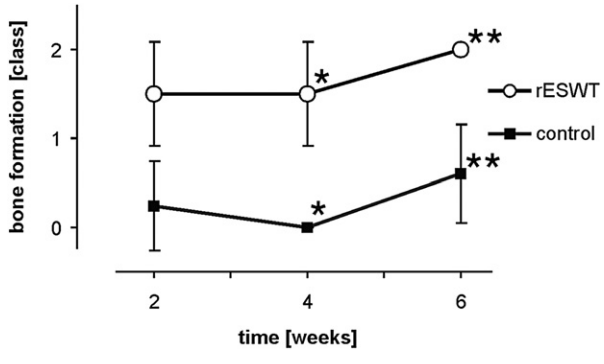

Fig. 3. Accumulated new bone formed at the ventral femoral cortex 2 to 6 weeks after the first radial extracorporeal shock wave therapy (rESWT) (rating system A). Stars indicate statistically significant differences (*$p = 0.029$; **$p = 0.008$).

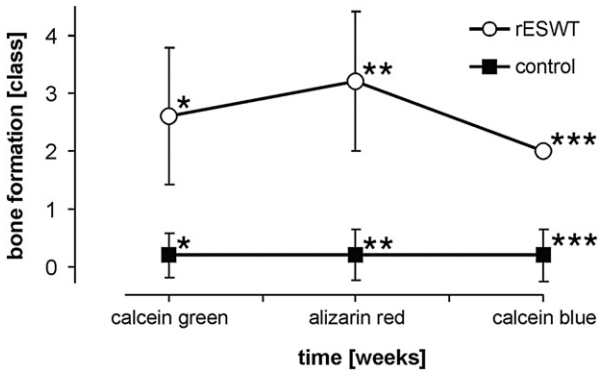

Fig. 4. Assessment of new bone formation 2 to 6 weeks after the first radial extracorporeal shock wave therapy (rESWT) (1 to 5 weeks after second rESWT) with evaluation of the single fluorochromes applied (means and standard deviations, rating system B). Stars indicate statistically significant differences (*$p < 0.0005$; **$p < 0.0005$; ***$p = 0.008$). Significant stimulation of bone formation was demonstrated already 2 weeks (calcein green) after the first rESWT with a peak of osteogenesis at 4 weeks (alizarin red) and a consecutive decline until the study end at week 6 (calcein blue).

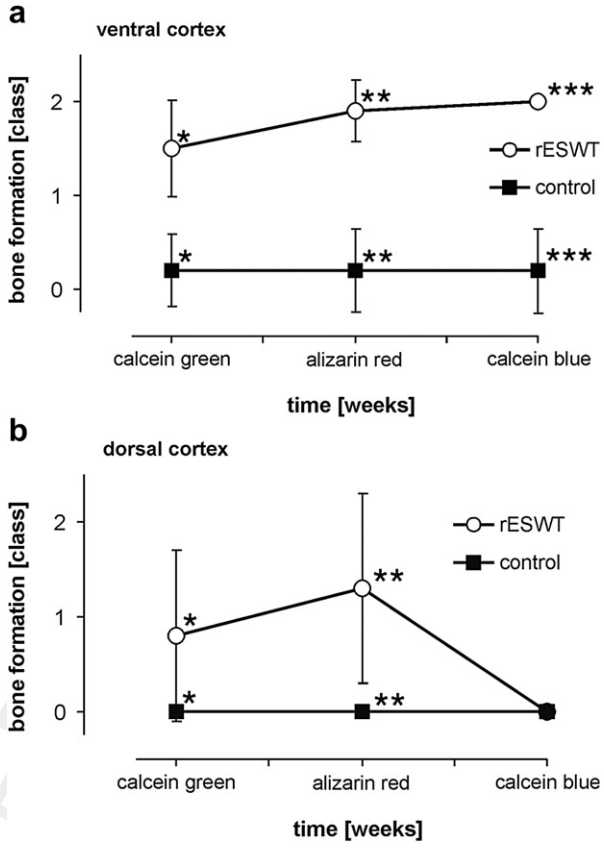

Fig. 5. New bone formation (a) at the ventral femoral cortex, and (b) at the dorsal femoral cortex at different time points 2 to 6 weeks after the first radial extracorporeal shock wave therapy (rESWT) represented by the corresponding bands of single fluorochromes (rating system B). Stars indicate statistically significant differences: (a) ventral femoral cortex: *$p < 0.0005$; **$p < 0.0005$; ***$p = 0.008$. (b) dorsal femoral cortex: *$p < 0.0005$; **$p < 0.0005$. Ossification declined at the dorsal cortex compared with the ventral cortex that was closely oriented to the shock wave device emphasizing the energy-dependent manner of new bone formation ($p = 0.008$, calcein blue).

maximum pressure at the wave front, followed by a lower tensile amplitude (Gerdesmeyer et al. 2002). Radial shock waves are missing the typical steepening effect of focused shock waves and, therefore, physically resemble simple pressure waves. The most important mechanical effects of shock waves are reflection with pressure and tension forces at borders of different impedances as well as the generation of cavitation bubbles in liquids, which induce shear forces by high velocity liquid streams ("jet-streams") (Delacretaz et al. 1995; Delius et al. 1998; Gerdesmeyer et al. 2002).

Mechanism of ESWT-induced new bone formation

Various studies have investigated the effect of focused shock waves on normal, osteotomized and fractured bone in different animal models and cell culture (Wang FS et al. 2002a; Maier et al. 2003a, 2003b; Chen et al. 2003).

Whereas the effectiveness of ESWT to stimulate bone healing after fracture is discussed controversially, the positive osteogenic effect on normal bone and bone defects has been proven. Wang and coworkers intensively studied shock wave induced reactions in bone on the molecular level and were able to reveal some of the basic principles (Wang FS et al. 2003; Chen et al. 2004a, 2004b). Thereby, two major mechanisms have been detected to be involved in the translation of mechanical shock wave energy to biologic responses: membrane hyperpolarization and the formation of free radicals. Wang et al. and Chen et al. demonstrated shock waves to induce hyperpolarization of cell membranes, followed by Ras activation and a local increase of stimulating factors like TGF-β1, VEGF-A and mitogen-activated protein kinases (MAPK) (Wang FS et al. 2003; Chen et al. 2004a). Consequently, increased proliferation and differentiation of mesenchymal stem cells to osteoblasts was observed. G-proteins of the cell membrane, which respond to mechanical stresses, were supposed to play a role in translating the kinetic energy of shock waves to Ras activation. Furthermore, shock waves were shown to produce oxygen radicals, which are also supposed to play a key role in connecting the mechanical shock wave energies and the resulting biological effects (Wang FS et al. 2001, 2003, 2004; Chen et al. 2004a). Wang et al. further showed that oxygen radical production was followed by a stimulation of a cascade of kinases and growth factors like VEGF, TGF-β1, BMP-1, BMP-2, BMP-7 *etc.*, followed by an increased growth and differentiation of mesenchymal cells toward osteoprogenitor

cells (Wang FS et al. 2002b, 2003, 2004; Chen et al. 2004a).

Dose-dependent effects

Dose-dependent stimulation of bone cells *in vitro* was observed by Kusnierczak et al. after shock wave application, with minimum threshold energy necessary to effect bone cell growth (Kusnierczak et al. 2000). However, bone cell stimulation was contributed to the total amount of energy applied, rather than single parameter like energy flux density or number of administered impulses. Furthermore, cell damage by excessive energy flux densities was described. Wang et al. and Chen et al. confirmed those findings *in vivo* proving a dose-dependent effect of ESWT on bone mass and bone strength in acute fracture healing in rabbits (Wang CJ et al. 2004) and in bone defect models in rats (Wang FS et al. 2002a; Chen et al. 2004b). Furthermore, suppression of osteogenetic influence was observed with the application of excessive energy levels. Maier et al. also provided data about deleterious effects of very high energy flux densities ($\geq$0.9 mJ/mm^2), demonstrating soft tissue edema, cortical fractures, periosteal detachment, intraosseous bleeding and even displacement of bone fragments to pulmonary vessels with the risk of pulmonary embolism (Maier et al. 2003a, 2003b, 2004). Apart from the studies with bone defects, other authors described osteostimulative effects with lower energies. Tischer et al. detected signs of new bone formation in areas located well outside the focus zone (Tischer et al. 2002).

Our study is the first proving a significant induction of new bone formation by rESWT, thereby applying low energy flux densities (0.16 mJ/mm^2) but relatively high impulse numbers (2 $\times$ 4000 impulses). Once induced, new bone formation persisted for at least 5 weeks after the last shock wave application. Bone growth was also activated at the dorsal femoral cortex in spite of the relatively low energy flux density, proving penetration of radial shock waves through soft tissue and bone. However, induction of new bone formation was significantly greater and lasted longer at the ventral cortex that had been directed toward the shock wave device, compared with the dorsal femoral cortex. These observations can be explained by a distance-related decline of shock wave energy while penetrating the thigh and confirm the dose-dependency of shock-wave induced osteogenesis. Consequently, the application of shock waves from different sides of the treated bone is recommended in the clinical setting to provide relevant energy levels to all cortices.

The significance of microtraumata like periosteal detachment and cortical and trabecular microfractures for the induction of osteogenesis has been discussed controversially (Delius et al. 1995; Haupt et al. 1992; Maier et al. 2004). In our qualitative analysis, we neither observed any histologically detectable traumata (like fractures, hematomas or periosteal detachments) nor fractures or callus formation detectable by microradiography. Our data are in accordance with results published by others demonstrating that cortical fractures and periosteal detachment are no prerequisites for new bone formation (Maier et al. 2002, 2004; Tischer et al. 2002). However, new bone formation was limited to endosteum and periosteum in our investigation, whereas other studies also demonstrated trabecular new bone formation related to trabecular microfractures (Delius et al. 1995). We conclude that iatrogenic fractures are not mandatory for periosteal and endosteal new bone formation; however, it remains to be clarified if microfractures provide an additional stimulus for new bone formation in cancellous bone.

Dynamic of ESWT-induced bone formation

In the treatment of bone pathologies, ESWT is usually repeated to complete three to six interventions with treatment free intervals ranging from 4–8 weeks (Gollwitzer et al. 2006). However, these recommendations are based on empirical clinical observations and not on controlled experimental data. In our study, osteogenesis was induced significantly by rESWT already within the first week after shock wave treatment. A peak of new bone formation was observed 4 weeks after the first rESWT with a consecutive decline of osteogenesis at week 6. The decline of new bone formation was most prominent at the dorsal femoral cortex, whereas increased bone formation persisted at the ventral cortex for at least 5 weeks after the last shock wave application. We, therefore, anticipate that both the intensity of new bone formation as well as its persistence over time is dose-dependent. Our results suggest repeating shock wave treatment after approximately 5–6 weeks, since a significant decline of new bone formation was observed after that period.

Interestingly, fluorescent microscopy also demonstrated inhomogeneous and weak bands of tetracycline in the rESWT treated bone, whereas no tetracycline deposition was observed in the control group. Thus, we anticipate that new bone formation was stimulated immediately after ESWT followed by integration of remaining circulating tetracycline that had been injected prior to shock wave treatment.

Abundant experimental and clinical evidence exist that mechanical stimuli can both positively and negatively influence fracture healing, bone regeneration and bone mass (Carter et al. 1988; Augat et al. 1996; Claes and Heigele 1999). Apart from focused shock waves, especially, cyclic loading and vibrational stimulation

have been abundantly investigated with positive effects on bone regeneration in fractured and normal bone (Fritton et al. 2005; Gardner et al. 2006). rESWT also has to be discussed in this context, since rESWT produces repeated mechanical stimuli by controlled compression and distension perpendicular to the treated bone, which can be easily applied in the clinical setting. Future studies have to show if rESWT is beneficial in the treatment and prevention of bone pathologies like osteoporosis and fracture nonunions.

We conclude that the osteogenetic effect of ESWT is a complex, dose-dependent biologic response persisting for several weeks after stimulation. rESWT has proven effective to induce new bone formation in normal bone and might be advantageous because of the application to larger treatment areas. However, limitations might be found in pathologies far below the skin level because of a distance related decline of shock wave energy. Nevertheless, rESWT might offer new perspectives in the therapy of bone pathologies as larger tissue areas can be effectively treated.

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