Calcif Tissue Int (2007) 81:124–131
DOI 10.1007/s00223-007-9046-9

Upregulation of VEGF in Subchondral Bone of Necrotic Femoral Heads in Rabbits with Use of Extracorporeal Shock Waves

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Received: 20 March 2007 / Accepted: 20 May 2007 / Published online: 16 July 2007

Abstract Extracorporeal shock wave treatment appears to be effective in patients with avascular necrosis of the femoral head. However, the pathway of biological events whereby this is accomplished has not been fully elucidated. The purpose of this study was to investigate the effect of extracorporeal shock waves on vascular endothelial growth factor (VEGF) expression in necrotic femoral heads of rabbits. VEGF expression was assessed by immunohistochemistry, quantitative real-time PCR, and Western blot analysis. The degree of angiogenesis was also assessed, as determined by the microvessel density (MVD), the assessment of which was based on CD31-expressing vessels. Bilateral avascular necrosis of femoral heads was induced with methylprednisolone and lipopolysaccharide in 30 New Zealand rabbits. The left limb (the study side) received shock wave therapy to the femoral head. The right limb (the control side) received no shock wave therapy. Biopsies of the femoral heads were performed at 1, 2, 4, 8, and 12 weeks. Western blot analysis and real-time PCR showed that shock wave therapy significantly increased VEGF protein and mRNA expression, respectively, in the subchondral bone of the treated necrotic femoral heads. Compared with the contralateral control without shock wave treatment, the VEGF mRNA expression levels increased to a peak at 2 weeks after the shock wave treatment and remained high for 8 weeks, then declined at 12 weeks, whereas the VEGF protein expression levels increased to a peak at 4 weeks after the shock wave treatment and remained high for 12 weeks. The immunostaining of VEGF was weak in the control group, and the immunoreactivity level in the shock-wave-treated group increased at 4 weeks and persisted for 12 weeks. The most intensive VEGF immunoreactivity was observed in the proliferative zone above the necrotic zone. At 4, 8, and 12 weeks after the shock wave treatment, MVD in subchondral bone from treated femoral heads was significantly higher than that in subchondral bone from untreated femoral heads. These data clearly show that extracorporeal shock waves can significantly upregulate the expression of VEGF. The upregulation of VEGF may play a role in inducing the ingrowth of neovascularization and in improving the blood supply to the femoral head.

Keywords Extracorporeal shock wave treatment · Osteonecrosis · Subchondral bone · Vascular endothelial growth factor (VEGF)

Interest in the use of extracorporeal shock waves to treat osseous afflictions was stimulated by the observation that shock waves that interacted with the pelvis during lithotripsy caused an increase in pelvic bone density [1]. In the field of orthopedics shock waves have been used for the therapy of delayed union or nonunion of fractures, calcifying tendonitis of the shoulder, lateral epicondylitis of the elbow, proximal plantar fasciitis, and Achilles tendonitis [2–8]. More recently, the results of shock wave therapy for avascular necrosis of the femoral head have been encouraging. Extracorporeal shock wave treatment appeared to be more effective than core decompression and nonvascularized fibular grafting in patients with early-stage osteonecrosis of the femoral head, although the mechanism of action remains unclear [9].

VEGF is a specific mitogenic factor for vascular endothelial cells, which stimulates endothelial cells

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proliferation, promotes neovascularization, and increases vascular permeability [10, 11]. Osteogenesis and angiogenesis occur in a coordinated manner in skeletal tissue and angiogenesis is an essential and tightly regulated process in bone formation and repair [12]. Extracorporeal shock wave treatment has previously been shown to affect local blood flow and bone metabolism in rabbit femur [13]. Thus we postulated that VEGF might be involved in the positive effects of shock wave therapy on avascular femoral head necrosis. The purpose of this study was to determine whether VEGF plays a role in neovascularization and repair of necrotic femoral heads treated with extracorporeal shock waves.

Materials and Methods

Experimental Design

The local Animal Care and Use Committee approved the following experimental study. Thirty New Zealand white rabbits with a body weight ranging from 3.0 to 4.0 kg were used in this study. Bilateral femoral head necrosis was induced in these animals with methylprednisolone and lipopolysaccharide according to Yamamoto et al. [14]. The left limb of the rabbit received shock wave therapy to the femoral head at 6 weeks after the injection of methylprednisolone and was designated the study side. The right limb received no shock wave therapy and was designated the control side. On the study side, the femoral head received 2,000 impulses and 0.26 mJ/mm^2 shock wave treatment using an Orthospec (Medispec, MD, USA). The shock wave dosage was selected on the basis of previous animal studies [15, 16]. The 30 animals were killed with an overdose of pentobarbital at 1 (n = 6), 2 (n = 6), 4 (n = 6), 8 (n = 6), and 12 (n = 6) weeks after shock wave therapy. Subchondral bone samples were obtained from the shock-wave-treated femoral heads and untreated femoral heads. Histology, immunohistochemistry, Western blot analysis, and quantitative real-time reverse transcriptase polymerase chain reaction were performed on the femoral heads from all 30 animals.

Rabbit Model of Avascular Necrosis of the Femoral Head

Avascular necrosis of the femoral head was induced with methylprednisolone and lipopolysaccharide as described previously [14]. The rabbits were given two intravenous injections of 10 μg/kg lipopolysaccharide at an interval of 24 hr and three intramuscular injections of 40 mg/kg methylprednisolone at intervals of 24 hr after the second injection of lipopolysaccharide. Avascular necrosis of the femoral head occurred at 6 weeks after the third injection of methylprednisolone.

Histology

The specimens were fixed in 10% neutral buffered formalin, decalcified using EDTA, embedded in paraffin, and cut into sections 4 mm thick in a coronal plane. Sections were stained with hematoxylin-eosin.

Immunohistochemistry

Deparaffinized sections were digested with 0.1% trypsin (Sigma, USA) and 0.1% $CaCl_2$ in phosphate-buffered saline (PBS) for 15 min at 37°C and washed with demonized water. Endogenous peroxidase activity was deactivated by immersing sections in 3% hydrogen peroxide in 0.01 M PBS for 10 min at 37°C, and rinsing several times in PBS. Sections were blocked with 10% bovine serum in PBS for 30 min at room temperature to reduce nonspecific binding, and incubated overnight at 4°C with a primary antibody. Primary antibodies against VEGF and CD31 (Upstate Biotechnology, Lake Placid, NY, USA) were used. The VEGF primary antibody is a mouse anti-rabbit antibody that recognizes 121, 165, and 189 amino acid isoforms of rabbit VEGF. A goat anti-mouse IgG biotinylated antibody diluted in a buffer (1:250) was used as a secondary antibody. Sections were further incubated with the biotinylated anti-mouse IgG and streptavidin peroxidases for 30 min, respectively, at 37°C. Then sections were thoroughly rinsed in PBS with shaking following each step. Immunoreactivity was determined by incubating the sections in a chromogen solution containing diaminobenzidine (DAB) and 0.1% hydrogen peroxide in the dark, followed by counterstaining with hematoxylin. The sections were dehydrated in alcohol and mounted using Permount. Negative control sections were processed using similar steps as described above but were incubated only with PBS or normal nonimmune mouse IgG.

Total RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction

After being quick-frozen in liquid nitrogen, the subchondral bone samples were stored at –80°C for protein and mRNA extraction later. The samples were pulverized with a mortar and pestle under liquid nitrogen in a RNase-free condition. Total RNA was extracted using Trizol reagent (Life Technologies, Grand Island, NY, USA) and precipitated in ethanol. The concentration of RNA was quantified by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer, and the purity of RNA was assessed by the ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}); in

addition the integrity and size distribution of RNA was checked by formaldehyde-agarose gel electrophoresis and ethidium bromide staining.

Reverse transcription followed by quantitative real-time polymerase chain reaction (qPCR) was performed as described previously. TaqMan primer and probe pairs for rabbit VEGF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control were based on database sequences and were designed using PrimerExpress Software (Applied Biosystems, Foster City, CA, USA). The probe sequences were: rabbit VEGF, CGTCACCATGCAGATCATGCG; rabbit GAPDH, GGTCGTG GACCTGACCTG; the 5′-end nucleotide of the probe was labeled with a reporter dye [FAM (6-carboxy- fluorescein)] and the 3′-end nucleotide of the probe was labeled with a quencher dye [TAMRA (6-carboxy-tetramethylrhodamine)]. The primer sequences were: rabbit VEGF forward, TGCCCACCGAGGAGTTCA; reverse, GGCCC TGGTGAGGTTTGAT; rabbit GAPDH forward, AAACT CACTGGCATGGCCTT; reverse, TTAGCAGCTTTCTCCAGGCG. Real-time qPCR was done in triplicate on the cDNA with an ABI 7300 Sequence Detector (Applied Biosystems) following the recommended protocols. Results were normalized to GAPDH levels using the formula: ΔCt (threshold cycle) = Ct of target gene – Ct of GAPDH. The comparative Ct method was used to investigate the amount of target gene relative to a calibrator. From the 30 animals, an arbitrary animal was selected as calibrator. The ΔΔCt value was calculated as follows: ΔΔCt of an animal = ΔCt of an animal – ΔCt of the calibrator. The amount of target gene normalized to GAPDH and relative to a calibrator of an animal was given by the formula $2^{-\Delta\Delta Ct}$. Then, the amounts of mRNA expression were compared between the shock-wave-treated femoral heads and the untreated controls. The fold change between the shock-wave-treated femoral head and untreated control was calculated as follows: Value of the shock-wave-treated femoral head/Value of the untreated control, at each time point. Fig. 3 demonstrates the fold change and its range for each experimental group when the untreated control at 1 week was normalized as 1.

Western Blot Analysis

For Western blots, proteins were extracted from the samples in a RIPA (radioimmunoprecipitation) buffer (8 times the sample volume) containing a protease inhibitor cocktail (Protease Inhibitor Cocktail Set I; Calbiochem, Darmstadt, Germany) at 4°C overnight. Extraction solutions were spun down at 14,000 rpm for 30 min in a refrigerated microcentrifuge. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% gels). A human recombinant VEGF (42 kDa; Chemicon International, Temecula, CA, USA) and SeeBlue Pre-stained Protein Standard (Invitrogen) were loaded as a positive control and molecular weight markers, respectively. Proteins were transferred to a PVDF membrane (Invitrogen), blocked with 2% bovine serum albumin (BSA), and exposed to a 1:100 dilution of the mouse antibody used for immunohistochemistry (Upstate Biotechnology, Lake Placid, NY, USA) in 1% BSA overnight. After washing in 0.1% Tween and 1× PBS buffer, the membrane was incubated with goat anti-mouse IgG horseradish peroxidase linked antibody (1:5,000) for 1 hr at room temperature and visualized using a nonradioactive ECL kit (Amersham Biosciences, Piscataway, NJ, USA). β-actin Western blots were performed on the same membrane after a ripping procedure. Samples from each animal were repeated twice, and the results were found to be the same.

Quantification of Microvessel Density

Microvessel density (MVD), a measure of angiogenesis, was determined by light microscopy after immunostaining sections with anti-CD31 antibodies according to the procedure described by Weidner et al. [41]. Microvessels were counted on a ×200 field. Any single endothelial cell or cluster of endothelial cells clearly separated from adjacent microvessels was considered as one countable microvessel. The MVD was counted in five representative areas of the section and the MVD was defined as the mean value from regions with different vessel numbers. The evaluation was performed by three persons, without knowledge of the group.

Statistical Analysis

The values for the shock-wave-treated femoral heads and untreated femoral heads were compared at each time point. Statistical differences between multiple groups were determined by one-way analysis of variance (ANOVA). A Student-Neuman-Keuls test was used to determine significance between two groups. A p value of <0.05 was considered statistically significant.

Results

Histologic Examination

Treated Group

At 1 week and 2 weeks after shock wave application, hemorrhage in the medullary cavity was observed (Fig. 1 A). At 4 weeks after shock wave application, capillary formation was prominent in the medulla and some osteo-

blasts were observed (Fig. 1B). At 8 weeks after shock wave application, significant immature bone and an increased number of osteoblasts were observed (Fig. 1C). At 12 weeks after shock wave application, thickened trabecular bone was observed and the trabecular bone was surrounded by a significant number of osteoblasts (Fig. 1D).

Control Group

At 1–4 weeks after shock waves were applied to the treated group, subchondral bone of the femoral heads in the control group showed sparser trabecular bone with empty lacunae or pyknotic nuclei of osteocytes, and the medulla exhibited fatty marrow (Fig. 1E). At 8 weeks vascular granulation tissue was seen invading the necrotic marrow space and osteoclasts were present at the neovascularization front actively resorbing the necrotic bone. Osteoblasts could also be seen at the surface of the trabecular bone. At 12 weeks trabecular bone became thin; some were broken.

Immunohistochemical Analysis

In the subchondral bone of the necrotic femoral heads not treated with shock waves, a slight VEGF immunoreactivity (VEGF-IR) was found in the osteoblasts and endothelial cells during the experiments (Fig. 2A). VEGF-IR was absent when the primary antibody was replaced with PBS or nonimmune mouse serum, suggesting specific binding of the VEGF antibody (Fig. 2F).

At 2 and 4 weeks after shock wave therapy, the osteoblasts and endothelial cells showed an increased level of VEGF-IR (Fig. 2B). At 8 weeks, VEGF-IR was increased in the fibroblastic and endothelial cells of the vascular granulation tissue invading the necrotic bone. Bone matrix and osteocytes also showed VEGF-IR (Fig. 2C). At 12 weeks, VEGF-IR was observed in the trabecular bone and the marrow. Osteoblasts adjacent to the newly formed woven bone showed intensive expression of VEGF. VEGF-IR was also observed in endothelial cells, osteocytes, and bone matrix (Fig. 2D).

VEGF Protein Expression

Positive human recombinant VEGF resolved a protein band at 42 kDa. The proteins extracted from the subchondral bone of the untreated control and shock-wave-treated femoral heads also formed a single VEGF-IR band between 40 and 45 kDa (Fig. 4). An increase in both band density and width was consistently observed in the subchondral bone samples obtained from the shock-wave-treated femoral heads at 2, 4, 8, and 12 weeks after the shock wave treatment, in comparison with the samples from the untreated femoral heads. In the samples obtained at 1 week

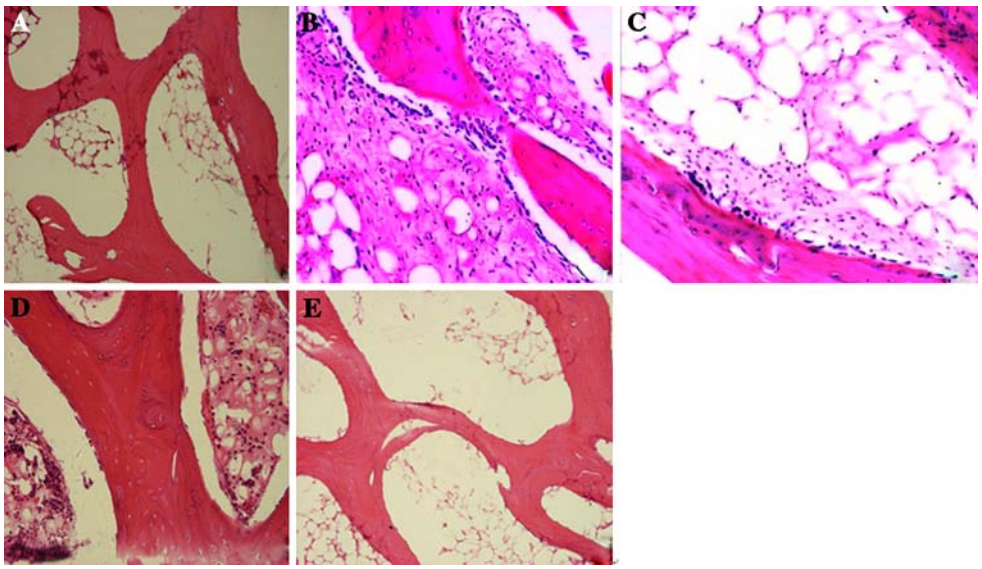

Fig. 1 A–E. Histologic findings of the femoral head following the application of shock wave treatment. **A** Mid-coronal section from the rabbit femoral head at 1 week after treatment, stained with hematoxylin-eosin. Treated femoral head at **B** 4 weeks, **C** 8 weeks, and **D** 12 weeks after shock wave treatment. **E** Untreated femoral head at 12 weeks. At 1 and 2 weeks after shock wave application (**A**), hemorrhage in the medullary cavity was observed (×100). At 4 weeks after shock wave application (**B**), capillary formation was prominent in the medulla and some osteoblasts were observed (×400). At 8 weeks after shock wave application (**C**), significant immature bone and increased number of osteoblasts were observed (×400). At 12 weeks after shock wave application (**D**), thickened trabecular bone was observed surrounded by a significant number of osteoblasts (×200). In the untreated femoral head (**E**) sparser trabecular bone with empty lacunae or pyknotic nuclei of osteocytes and fatty marrow in the medulla were observed (×100)

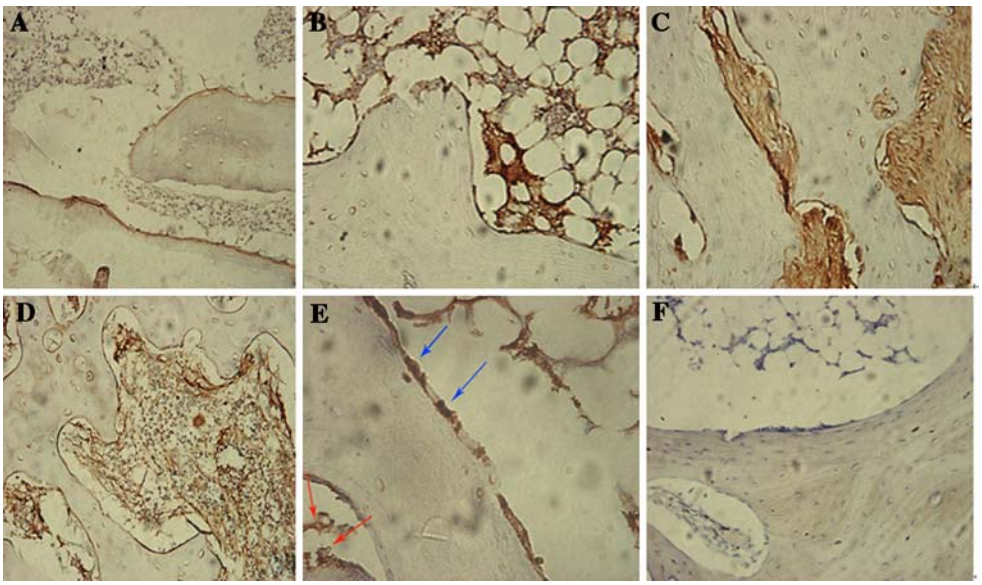

Fig. 2 A–F. Photomicrographs of the subchondral bone immunostained with anti-VEGF. **A** In the necrotic femoral heads not treated with shock waves, a slight VEGF immunoreactivity (VEGF-IR) was found in the osteoblasts and endothelial cells during the experiments (×200). **B** At 4 weeks after shock wave application, the osteoblasts and endothelial cells showed an increased level of VEGF-IR (×200). **C** At 8 weeks after shock wave application, VEGF-IR was increased in the fibroblastic and endothelial cells of the vascular granulation tissue invading the necrotic bone. The bone matrix and osteocytes also showed VEGF-IR (×200). **D** At 12 weeks after shock wave application, osteoblasts adjacent to the newly formed woven bone showed intensive expression of VEGF. VEGF-IR was also observed in endothelial cells, osteocytes, and bone matrix (×200). **E** In a higher magnification image, osteoblasts (blue arrows) and endothelial cells (red arrows) show intense VEGF-IR (×400). **F** VEGF-IR was absent when the primary antibody was replaced with PBS or nonimmune mouse serum (×200)

after the shock wave treatment there was no difference between the shock-wave-treated femoral heads and the untreated femoral heads.

VEGF mRNA Expression

The gene expression of VEGF was markedly higher in the shock-wave-treated femoral heads compared with the untreated femoral heads at all time points. Statistically significant differences were observed at 1, 2, 4, and 8 weeks after shock wave treatment. The maximum fold change was 68 at 2 weeks after shock wave treatment (Fig. 3).

MVD in Subchondral Bone of Femoral Heads

There were only a few microvessels in the subchondral bone of the necrotic femoral heads not treated with shock waves. When the MVD of the femoral heads treated with shock waves was compared with that of the femoral heads not treated with shock waves, significantly higher numbers of microvessels in the subchondral bone were present in the treated femoral heads at 4, 8, and 12 weeks after the shock wave treatment. MVD did not differ significantly between the two groups of femoral heads at 1 and 2 weeks after shock wave treatment (Fig. 5).

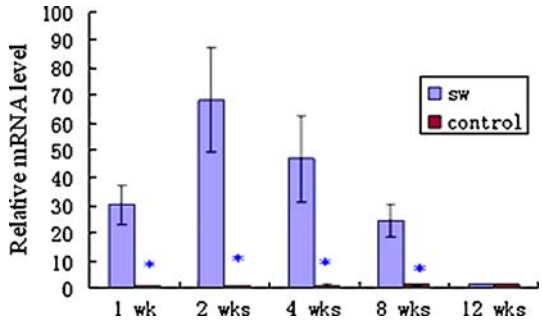

Fig. 3 Gene expression of VEGF in shock-wave-treated femoral heads (blue bars) and in untreated femoral heads (red bars) at 1, 2, 4, 8, and 12 weeks after the application of shock wave treatment analyzed by real-time quantitative RT-PCR. The graph demonstrates the fold change and its range of each experimental group when the untreated femoral head at 1 week was normalized as 1. The asterisks indicate statistically significant differences between the shock-wave-treated femoral head and the untreated femoral head at the indicated time point. The superscripts show the maximum fold change. SW, shock-wave-treated femoral heads; control, untreated femoral heads; Wk, week

Discussion

Many methods have been tried for the treatment of avascular femoral head necrosis, such as conservative treatment of with analgesics and non-weight-bearing, core decom-

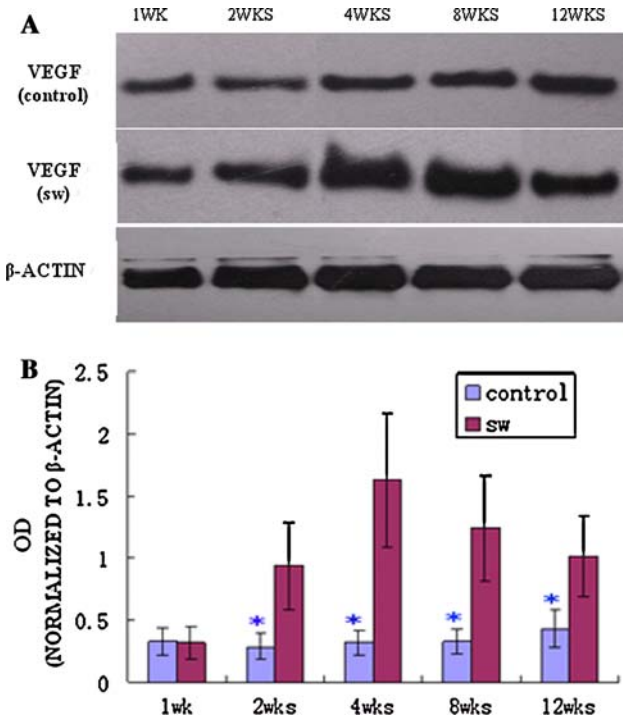

Fig. 4 **A** Western blot analysis of the subchondral bone samples obtained from the necrotic femoral heads at 1, 2, 4, 8, and 12 weeks after the application of shock wave treatment (lanes 1WK, 2WKS, 4WKS, 8WKS, and 12WKS represent 1, 2, 4, 8, and 12 weeks, respectively). Western blot analysis consistently showed increased VEGF at 2, 4, 8, and 12 weeks in comparison with the samples from the untreated control femoral heads. **B** The optical density analysis of the Western blot, normalized to the optical density of β-actin in each lane. The asterisks indicate statistically significant differences between the shock-wave-treated femoral heads and the untreated femoral heads at the indicated time point. The superscripts show the maximum fold change. SW, shock-wave-treated femoral heads; control, untreated femoral heads; Wk, week

pression with or without bone grafting, intertrochanteric osteotomy aimed at rotating the necrotic focus out of the zone of loading, bone transplants with vascular pedicles, and total hip replacement after the failure of head-preserving methods. Most treatment methods have demonstrated limited effects in selected series, but none has shown universal success [17–23]. Shock wave treatment is a new therapeutic modality that has shown promise as a nonsurgical treatment for avascular femoral head necrosis [24–26]. However, the mechanism by which shock wave treatment results in clinical improvement remains uncertain. Some have postulated that shock wave therapy provokes a painful level of stimulation, and relieves pain by hyperstimulation analgesia, while others have speculated that shock wave therapy produces microfracture and activates cells to express genes for osteogenesis which in turn cause new bone formation [27–30]. The results of this study have shown that VEGF mRNA expression is upregulated as early as 1 week after shock wave treatment, and that the upregulation is maintained at 2, 4, and 8 weeks after shock wave treatment. Western blot experiments verified these findings at the protein level. The VEGF protein expression levels increased to a peak at 4 weeks after the shock wave treatment and remained high for 12 weeks. It is important to note that the most intensive VEGF immunoreactivity was observed in the proliferative zone above the necrotic zone.

Angiogenesis is an essential component of skeletal development and repair. The process of bone development and repair depends on adequate formation of new capillaries from existing blood vessels [12, 31]. Several growth factors and cytokines have demonstrated the ability to induce angiogenesis, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), insulin growth factor-1 (IGF-1), and epidermal growth factor (EGF) [32–35]. Among these factors VEGF plays a central role in normal and abnormal angiogenesis [36]. In vitro and in vivo studies have demonstrated that VEGF can promote endothelial cell survival, proliferation, migration, proteolytic activity, branching morphogenesis, and vasodilation [10, 11, 37]. VEGF is a mitogen for vascular endothelial cells and is involved in the development and the formation of vessels [38]. Our data showed that VEGF expression was upregulated significantly in rabbit necrotic femoral heads treated with extracorporeal shock waves. At 4, 8, and 12 weeks after shock wave treatment, the density of microvessels in femoral heads treated with shock waves was also upregulated compared with that in femoral heads not treated with shock waves. A high density of microvessels was found at sites of new bone formation, with continuous rims of osteoblasts along the trabecular bone at 12 weeks after shock wave treatment. This indicates that angiogenesis plays an important role during new bone formation. Furthermore, because VEGF is essential for bone formation [40], it is possible that it may act as a key regulator that couples angiogenesis, bone formation, and repair of necrotic subchondral bone in femoral heads treated with shock waves.

The results of this study are in agreement with those of Wang et al. [39] who showed that shock wave therapy induces the ingrowth of neovascularization associated with early release of angiogenesis-related markers in the Achilles tendon-bone in rabbits. VEGF is the key regulator for angiogenesis and the resulting processes of bone formation. It is therefore highly likely that the positive effect of high-energy shock waves on VEGF expression in necrotic femoral heads contributes to the mechanism by which the shock waves increase the blood supply of necrotic femoral heads and promote repair of necrotic subchondral bone.

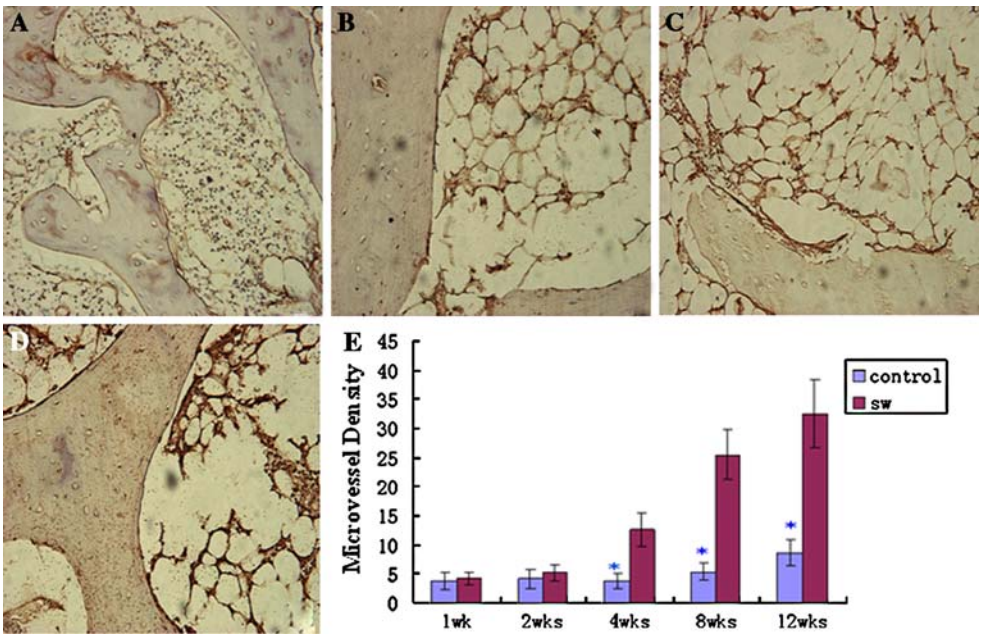

Fig. 5 A–E. Photomicrographs of the subchondral bone immunostained with anti-CD31. **A** There were only few microvessels in the subchondral bone of the necrotic femoral heads not treated with shock waves (×200). **B** Immunohistochemical staining, showing increased density of microvessels in a femoral head at 4 weeks after shock wave treatment (×200). **C** Immunohistochemical staining, showing increased density of microvessels in a femoral head at 8 weeks after shock wave treatment (×200). **D** A high density of microvessels was found at sites of new bone formation, with continuous rims of osteoblasts along the trabecular bone at 12 weeks after shock wave treatment (×200). **E** The MVD of the shock-wave-treated femoral heads (red bars) and the untreated femoral heads (blue bars). The asterisks indicate statistically significant differences between the shock-wave-treated femoral heads and the untreated femoral heads at the indicated time point. The superscripts show the number of microvessels. SW, shock-wave-treated femoral heads; control, untreated femoral heads; Wk, week

Acknowledgments This work was supported by a grant from the National Natural Science Foundation of China (30571898).

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