Shockwaves Enhance the Osteogenetic Gene Expression in Marrow Stromal Cells from Hips with Osteonecrosis

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Background: This in vitro study investigated the angiogenesis and osteogenesis effects of shockwaves on bone marrow stromal cells (BMSCs) from hips with osteonecrosis.

Methods: BMSCs were harvested from the bone marrow cavity of the proximal femur in six patients with osteonecrosis of the femoral head. The specimens were divided into four groups, the control, shockwave, shockwave plus nω-nitro-L-arginine methyl ester (L-NAME) and a nitric oxide (NO) donor (NOC18) groups. The control group received no shockwaves and was used as the baseline. The shockwave group received 250 shockwave impulses at 14 Kv (equivalent to 0.18 mJ/mm^2 energy flux density). The shockwave plus L-NAME group was pre-treated with L-NAME before receiving shockwaves. The NOC18 group received NOC18 after cell culture for 48 hours. The evaluations included cell proliferation (MTT) assay, alkaline phosphatase, real time reverse transcriptase-polymerase chain reaction analysis of vessel endothelial growth factor (VEGF), bone morphogenic protein (BMP)-2, RUNX2 and osteocalcin mRNA expression and von Kossa stain for mineralized nodules.

Results: The shockwave group showed significant increases in MTT, VEGF, alkaline phosphatase, BMP2, RUNX2 and osteocalcin mRNA expression and more mature mineralized nodules compared with the control. Pre-treatment with L-NAME significantly reduced the angiogenic and osteogenic effects of extracorporeal shockwave therapy (ESWT) and the results were comparable with the control. Administration of NOC18 significantly enhanced the angiogenesis and osteogenesis effects compared with the control and the results were comparable with the shockwave group.

Conclusion: ESWT significantly enhanced the angiogenic and osteogenic effects of BMSCs mediated through the NO pathway in hips with osteonecrosis. These innovative findings, at least in part, explain some of the mechanism of shockwaves in osteonecrosis of the hip.

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Key words: shockwave, bone marrow stromal cells, angiogenesis, osteogenesis, hip osteonecrosis

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The etiology of osteonecrosis of the femoral head (ONFH) is multi-factorial and includes steroid administration, alcohol abuse, traumatic events, vascular injury and idiopathic origins among others.[1-5] Treatment of ONFH is stage-dependent.[6-8] Conservative treatment with non-steroid anti-inflammatory drugs, physical therapy and protected weight bearing are recommended for the early stages, but the results are generally unsatisfactory.[6,7] Surgical intervention such as core decompression, non-vascularized or vascularized bone grafts, muscle pedicle grafts and derotational osteotomy are often indicated in symptomatic hips, however, the results are inconsistent and unpredictable.[9-12] Many patients eventually require total hip replacement, but this approach is prone to complications, particularly in young patients.[13,14]

Shockwaves are high-energy acoustic waves generated by either electrohydraulic or electromagnetic principles. In clinical application, extracorporeal shockwave therapy (ESWT) has been shown effective in the treatment of non-union of long bone fractures, and tendinopathy of the shoulder, elbow, knee and heel. In animal experiments, ESWT was shown to promote bone healing and tissue repair with ingrowth of neovascularization and upregulation of angiogenic and osteogenic growth factors such as vessel endothelial growth factor (VEGF), endothelial nitric oxide synthase (eNOS), proliferating cell nuclear antigen (PCNA), bone morphogenic protein-2 (BMP-2) and osteocalcin.[15] Prior studies demonstrated the effectiveness of ESWT in the treatment of hip necrosis.[16,17] Recent study showed the regeneration effects of ESWT in hips with osteonecrosis of the femoral head including angiogenesis, osteogenesis and bone remodeling.[18] However, the mechanism of ESWT in hips with osteonecrosis is poorly understood. The tissue actually targeted by ESWT remains unknown.

Bone marrow stromal cells (BMSCs) are multipotential progenitor cells that may enhance angiogenesis and osteogenesis under mechanical stimulation or appropriate culture conditions.[19] ESWT was shown to promote the growth and differentiation of bone marrow stromal cells toward osteoprogenitors associated with induction of transforming growth factor (TGF)-β1.[20] We hypothesized that BMSCs may play an important role in hips with osteonecrosis, and ESWT may enhance the angiogenesis and osteogenesis effects via nitric oxide mediation in hips with osteonecrosis. The purpose of this study was to investigate the effects of ESWT on the angiogenic and osteogenic gene expression of bone marrow stromal cells from hips with osteonecrosis in the presence of a nitric oxide (NO) synthase inhibitor or nitric oxide donor.

METHODS

The Institutional Review Board of our hospital approved this study, and all patients signed an informed consent prior to participation in the study.

Fifteen milliliters of blood were obtained from the bone marrow cavity of the proximal femur of six patients undergoing hip replacement surgery for osteonecrosis of the femoral head. After centrifugation of the blood sample at 500 xg for 30 min, the nucleated cells were harvested from the interface of the Ficoll-Paque density gradient (density, 1.007 g/ml; Pharmacia Biotech AB, Uppsala, Sweden). The nucleated cells were seeded in Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum for 30 min to separate adherent macrophages from non-adherent cells containing the stromal elements. The non-adherent cells were collected and plated (5×10^5 cell per well, 6-well plate). Twenty-four hours later, all non-adherent cells were discarded by two vigorous washings with PBS and the remaining adherent stromal cells were harvested by trypsinization. Flow cytometric characterizations of the stromal cells were performed. The characteristics of the BMSCs were confirmed with positive results for cluster of differentiation (CD)105, CD73 and CD90, and negative results for CD34 and CD14, and most cells were clustered in distribution. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of angiogenic and osteogenic gene expression was performed.

The BMSCs were divided into four groups, the control, shockwave, shockwave plus L-NAME (Nω-Nitro-L-arginine methyl ester hydrochloride, Sigma Inc, St. Louis, MO, U.S.A.), an NO synthase inhibitor, and NOC18 2'-(hydroxynitrosohydrazino) bis-ethanamine, A.G. Scientific Inc, San Diego, CA, U.S.A.), an NO donor. The control group received no shockwave treatment and served as the baseline. The shockwave group received shockwave treatment alone. The shockwave + L-NAME group was pre-

treated with L-NAME (100 ug/ml) for 1 hour before receiving shockwave treatment. The NOC18 group received NOC18 (10 ng/ml) after cell culture for 48 hours.

Application of shockwaves

A total of 1×10^6 harvested BMSCs were placed in a 1.5 ml Eppendorf container after mixing with 1.5 ml of medium until the container was completely filled. The source of the shockwaves was an OssaTron (Sanuwave, Alpharetta, GA, U.S.A.). The shockwave tube was centered on the container, and the depth of treatment was set at 0.5 cm. Shockwaves were applied in two sessions. The first session consisted of the application of 250 shockwave impulses at 14 Kv (equivalent to 0.18 mJ/mm^2 energy flux density) to the cells in the container (Fig. 1). The cells in the container were then shaken and mixed to ensure even cell distribution within the container before receiving the second session of shockwaves. The second session consisted of the application of 250 shockwave impulses at 14 Kv to the BMSCs within the container.

The BMSCs from the four groups were subjected to investigations including cell proliferation by 3-(4,5-dimethylthiazol-2yl) 2,5-diphenyl tetrazolium bromide (MTT) assay, alkaline phosphatase, real time RT-PCR analysis for VEGF, BMP-2, RUNX2 and osteocalcin mRNA expression, and von Kossa staining for mineralized nodules.

Cell proliferation (MTT) and angiogenesis

Cell proliferation was evaluated by MTT assay (Roche Diagnostics, Mannhein, Germany). Cells (1×10^4 cells/well, 96-well plate) were added to 10 μl of MTT per well and incubated for 4 hours. Formazan synthesized by cell cultures was solubilized using 100 μl 10% SDS-0.01M HCl and colorimetrically measured at 550 nm (Amersham, Buckinghamshire, U.K.).

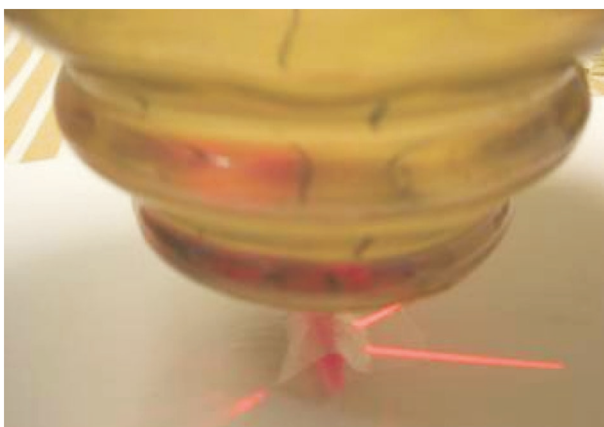

Fig. 1 The photograph shows an Eppendorf container with bone marrow stromal cells being subjected to ESWT.

Real time RT-PCR analysis for VEGF, BMP-2, RUNX-2 and osteocalcin

The induction of angiogenesis and osteogenesis growth factors including VEGF, BMP-2, RUNX-2 and osteocalcin mRNA expression were assessed by real-time RT-PCR. A total of 1 μg RNA harvested from cell cultures by QIAzol reagent (Qiagene Inc., Valencia, CA, U.S.A.) was reverse transcribed into cDNA for PCR amplification. The PCR reactions were initially run at 95°C for 3 min followed by 40 cycles at 95°C for 10 sec, 55°C for 45 sec and 95°C for 1 min using 2X SYBR Green Assay Kits (SuperArray Bioscience Co, Frederick, MD, U.S.A.) and the iCycler iQ® Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The sequence detection system and the arbitrary intensity threshold (Ct) of amplification were computed according to manufacturer instructions. Primer oligonucleotide sequences were used as follows: VEGF (forward: 5'-CCC ACT GAG GAG TCC AAC AT-3' and reverse: 5'-TTA TAC CGG GAT TTC TTG CG-3'); BMP-2 (forward: 5'-CCC CCT ACA TGC TAG ACC TGT-3' and reverse: 5'-CAC TCG TTT CTG GTA GTT CTT CC-3'), RUNX-2 (forward: 5'-GAA TGC TTC ATT CGC CTC ACA-3' and reverse: 5'-TGA CCT GCG GAG ATT AAC CAT-3'); osteocalcin (forward: 5'-AAG AGA CCA AGG CGC TAC CT-3' and reverse: 5'-GCC GAT AGG CCT CCT TGA AAG-3'); and glyceraldehyde 3-phosphate dehydrogenase GAPDH (forward, 5'-AAA TCC CAT CAC CAT CTT CC-3'; reverse, 5'-AGG CTG TTG TCA TAC TTC TC-3'). The Ct of amplification was computed. The relative gene expression level was $2^{(-\Delta Ct)}$, where $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{GADPH}}$. In cell culture experiments, the fold change in mRNA expression treatment was defined as $2^{-\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct_{\text{treatment}} - \Delta Ct_{\text{vehicle}}$.

Alkaline phosphatase

Aliquots of cultured supernatants were incubated with substrate buffer containing 50 mM glycine, 1

mM magnesium chloride (pH = 10.5) and 2.5 mM *p*-nitrophenyl phosphate (Sigma). The reactions were incubated at 37°C for 30 min, and then stopped using 0.1 ml of 1 N sodium hydroxide. The results were read at OD 405 nm on a micro-plate reader.

von Kossa stain

Cells (5 x 10^4 cells per well, 24-well plate) were cultured in osteogenic medium containing DMEM, 20% fetal bovine serum, 0.01 M dexamethasone, 10 mM -glycerophosphate and 50 μg/ml ascorbic acid (Sigma-Aldrich, St Louis, MO, U.S.A.) in a 5% CO2 incubator at 37°C for 21 days. The medium was changed every 3 days. On day 21, the culture medium was removed and cells were fixed with 4% formaldehyde in PBS buffer. The mineralized nodules in the cell cultures were detected by von Kossa stain.

Statistical analysis

All values are expressed as mean ± standard error. Parametric analysis of variance (ANOVA) and least significant difference (LSD) post hoc tests were used to assess the differences among groups. The level of statistical significance was set at $p < 0.05$.

RESULTS

The results for MTT and VEGF are shown in Table 1. At 48 hours after EWST, there were significant increases in MTT compared with the control ($p < 0.0001$). After pre-treatment with L-NAME, the proliferation effects of ESWT were abolished with the results comparable to the control ($p = 0.0566$). The differences in the increase of MTT between the shockwave group and the shockwave plus L-NAME group were statistically significant favoring the shockwave group ($p = 0.0002$). Administration of NOC18 significantly increased the cell proliferation compared with the control ($p = 0.0496$).

The VEGF expression was significantly elevated in the shockwave group compared with the control ($p = 0.0154$). Pre-treatment with L-NAME blocked the effects of ESWT on VEGF expression with results comparable to the control ($p = 0.0945$), whereas administration of NOC18 significantly increased the VEGF expression compared with the control ($p = 0.006$).

The results of alkaline phosphate measurements are shown in Table 2. At 72 hours after EWST, the level of alkaline phosphatase was significantly elevated compared with the control ($p = 0.001$). Pre-treatment with L-NAME significantly decreased the alkaline phosphatase level after ESWT with results comparable to that of the control ($p = 0.0867$). Administration of NOC18 significantly elevated the alkaline phosphatase level compared with the control ($p = 0.003$).

The results of BMP-2, RUNX-2 and osteocalcin mRNA expression on real time RT-PCR are shown in Table 3. The levels of expression of BMP-2, RUNX-2 and osteocalcin mRNA were significantly elevated after ESWT compared with the control ($p < 0.05$). Pre-treatment with L-NAME significantly decreased BMP-2, RUNX-2 and osteocalcin mRNA expression after ESWT with results comparable to that of the control ($p > 0.05$). Administration of NOC18 significantly increased the osteogenic markers including BMP-2, RUNX-2 and osteocalcin mRNA expression ($p < 0.05$).

Table 1. MTT (Cell Proliferation) and VEGF Expression

	Control	Shockwave	SW + L NAME	NOC18
MTT at 48 hrs	0.311 ± 0.005	0.352 ± 0.006	0.325 ± 0.006	0.348 ± 0.003
		p1: < 0.0001	*p2*: 0.0566	*p4*: 0.0496
			p3: 0.0002	
VEGF	1.0	4.9 ± 0.38	2.26 ± 0.64	4.52 ± 0.12
		p1: 0.0154	*p2*: 0.0945	*p4*: 0.0006
			p3: 0.0252	

Abbreviations: Values are expressed as mean ± SE; *p1*: Control VS Shockwave; *p2*: Control VS Shockwave + L NAME; *p3*: Shockwave VS Shockwave + L NAME; *p4*: Control VS NOC18; Parametric ANOVA and LSD post hoc tests were used to assess the differences among groups. The level of statistical significance was set at $p < 0.05$.

Table 2. Alkaline Phosphatase Levels (mMol/ml) at 72 hours

	Control	Shockwave	SW + L NAME	NOC18
72 hrs	1.537 ± 0.152	2.442 ± 0.156 *p1*: 0.001	1.996 ± 0.138 *p2*: 0.0867 *p3*: 0.0065	2.438 ± 0.042 *p4*: 0.003

Abbreviations: Values are expressed as mean ± SE; *p1*: Control VS Shockwave; *p2*: Control VS Shockwave + L NAME; *p3*: Shockwave VS Shockwave + L NAME; *p4*: Control VS NOC18; Parametric ANOVA and LSD post hoc tests were used to assess the differences among groups. The level of statistical significance was set at $p < 0.05$.

Table 3. BMP-2, RUNX-2 and Osteocalcin Levels

	Control	Shockwave	SW + L NAME	NOC18
BMP2	1	1.57 ± 0.18 *p1*: 0.0424	0.83 ± 0.12 *p2*: 0.1499 *p3*: 0.0161	1.46 ± 0.09 *p4*: 0.0016
RUNX2	1	1.6 ± 0.17 *p1*: 0.0371	0.93 ± 0.03 *p2*: 0.0918 *p3*: 0.0283	1.97 ± 0.19 *p4*: 0.0065
OCN	1	2.03 ± 0.3 *p1*: 0.0366	0.93 ± 0.03 *p2*: 0.918 *p3*: 0.0319	2.73 ± 0.29 *p4*: 0.0134

Abbreviations: Values are expressed as mean ± SE; *p1*: Control VS Shockwave; *p2*: Control VS Shockwave + L NAME; *p3*: Shockwave VS Shockwave + L NAME; *p4*: Control VS NOC18; Parametric ANOVA and LSD post hoc tests were used to assess the differences among groups. The level of statistical significance was set at $p < 0.05$.

The results of the von Kossa stain are shown in Fig. 2. Significantly more mineralized nodules were observed in the plates treated with ESWT alone and NOC18 compared with the control. Pre-treatment with L-NAME significantly reduced the mineralized nodules with the gross appearance of mineralization comparable to that of the control.

DISCUSSION

The results of the current study showed that ESWT upregulated VEGF, alkaline phosphatase, RUNX2 and BMP2 gene expression in bone marrow stromal cells from hips with osteonecrosis through the induction of the NO pathway. Some studies have shown that ESWT promotes osteogenesis in soft tissue, bone marrow and osteoblast-like cells.[21-25] Other studies demonstrated that ESWT induces the ingrowth of neovascularization and upregulation of angiogenesis and osteogenesis growth factors including induction of eNOS, VEGF, PCNA and BMP-2.[26,27] NO is an early mediator of bone formation by mechanical stimulation.[28] Some studies reported that

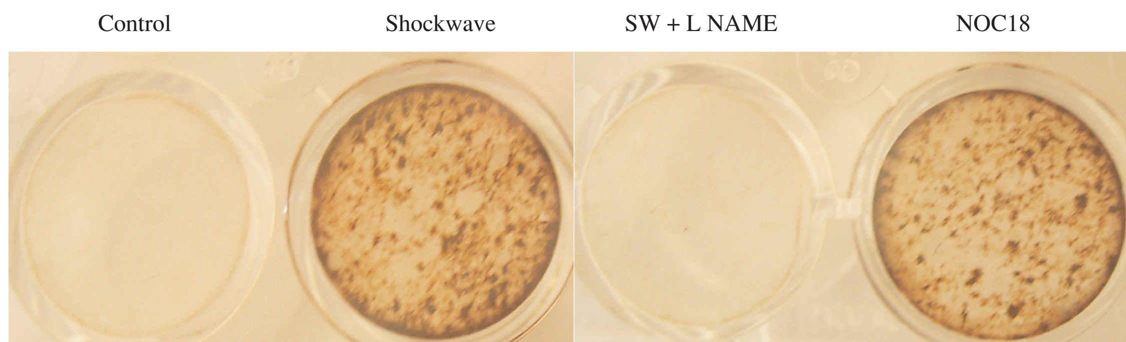

Fig. 2 Photographs show mineralized nodules in the control, ESWT, ESWT plus L-NAME and NOC18 groups.

NO increases osteoprotegerin and osteoclastogenesis-inhibitory activity in bone marrow stromal cells in ovariectomized rats,[29] and mediates factor-1α activation and VEGF-A expression in human osteoblasts.[30] Others showed that ESWT promotes growth and differentiation of bone-marrow stromal cells towards osteoprogenitors associated with induction of TGF-β1.[20] The increased angiogenesis, osteogenesis and bone remodeling after ESWT in patients with osteonecrosis of the hip does not necessarily mean there are no risks of bone resorption, or loss of mechanical integrity, and may actually predispose to subchondral fracture and failure of the hips from the disease. This may explain in part why ESWT is effective in some patients, but not in others in the clinical setting. It appears that bone marrow stromal cells play an important role when ESWT is utilized to treat hips with osteonecrosis of the femoral head.

Bone marrow contains abundant multi-potential progenitor cells that can be expanded ex vivo under mechanical stimulation and appropriate culture conditions, differentiate into various cell types, and enhance angiogenesis and osteogenesis.[19] Some studies showed that ESWT enhances recruitment of endothelial progenitor cells in chronic hind limb ischemia.[31] Our previous study showed that ESWT stimulated oxygen radical-mediated osteogenesis of mesenchymal cells from human umbilical cord blood.[32] The principal findings of this in vitro study demonstrated that ESWT promoted gene expression in angiogenesis and osteogenesis of bone marrow stromal cells from hips with osteonecrosis which was involved in the NO synthase pathway. An increase in VEGF could be an indication of increased vascular permeability and microvascular activity including angiogenic growth of new blood vessels. The formation of new vessels and bone may replace the lesion within the femoral head in total or in part depending on the effects of angiogenesis and osteogenesis after ESWT. This is reflected in clinical observation, in that some lesions regress, while others progress after treatment.[16]

This study was limited to the in vitro experiment design. The study samples were obtained only from patients with osteonecrosis of the femoral head. Although the results appear very impressive, we cannot be sure that a combination of bone marrow stromal cells and ESWT provide clinical benefits in patients with osteonecrosis of the femoral head. We are not sure whether similar effects exist in bone marrow stromal cells in patients without ONFH. Therefore, the clinical implications of this new concept rely on the results of future in vivo experiments.

In conclusion, extracorporeal shockwave enhances angiogenesis and osteogenesis gene expression in bone marrow stromal cells from hips with osteonecrosis through the NO-mediated pathway. The potential clinical implications of the in vitro results will rely on future in vivo experiments.

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體外震波增進股骨頭壞死症骨髓幹細胞在試管中的新生血管及骨骼增生效果

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背 景： 骨髓幹細胞在股骨頭壞死症扮演重要角色，本實驗研究體外震波對股骨頭壞死症的骨髓幹細胞產生的新生血管及骨骼增生的效果。

方 法： 骨髓幹細胞取自六位病人因股骨頭壞死而接受髖關節置換手術，摘取處置後的骨髓幹細胞分成四組；第一組是對照組 (Control)，沒有接受震波治療，第二組接受震波治療，第三組是接受震波加 L-NAME (NO inhibitor)，第四組接受 NOC18 (NO promotor)。評估分法包括 MTT (Cell proliferation), alkaline prosphatase 及 real time RT-PCR for VEGF, BMP-2, RUNX2, osteocalcin 以及 Von Kossa stain。

結 果： 在震波組，骨髓幹細胞的血管新生及骨骼增生 (MTT, VEGF, alkaline phosphalase, BMP2, RUNX2, osteocalcin 以及礦物化結等) 都有顯著增加，達到統計上的意義。在震波加 L-NAME 組，體外震波對骨髓幹細胞的作用全被 L-NAME 抵銷，結果和對照組類同。加入 NOC18 後，骨髓幹細胞的血管新生及骨骼增生明顯增加，與打體外震波的結果類同。

結 論： 體外震波經由 NO pathway 的媒介對股骨頭壞死症的骨髓幹細胞刺激血管新生及骨骼增生。這些研究成果至少可以部分解釋體外震波對股骨頭壞死的治療機轉。

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關鍵詞： 體外震波，骨髓幹細胞，血管新生，骨骼增生，股骨頭壞死

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