Nitric Oxide 20 (2009) 298–303

Contents lists available at ScienceDirect

Nitric Oxide

journal homepage: www.elsevier.com/locate/yniox

The effects of shockwave on bone healing and systemic concentrations of nitric oxide (NO), TGF-β1, VEGF and BMP-2 in long bone non-unions

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ARTICLE INFO

Article history:
Received 1 October 2008
Revised 19 January 2009
Available online 10 March 2009

Keywords:
Shockwave
Non-union
Bone healing
Nitric oxide (NO)
TGF-β1
VEGF
BMP-2

ABSTRACT

This study investigated the effects of extracorporeal shockwave treatment (ESWT) on bone healing and the systemic concentrations of nitric oxide (NO), TGF-β1, VEGF and BMP-2 in long bone non-unions. Forty-two patients with 42 established non-unions of the femur and tibia were enrolled in this study. Each long bone non-union was treated with 6000 impulses of shockwave at 28 kV in a single session. Ten milliliters of peripheral blood were obtained for measurements of serum NO level and osteogenic growth factors including TGF-β1, VEGF and BMP-2; serum levels of calcium, alkaline phosphatase, calcitonin and parathyroid hormone before treatment and at 1 day, 1, 3 and 6 months after treatment. The evaluations for bone healing included clinical assessments and serial radiographic examinations. At 6 months, bony union was radiographically confirmed in 78.6%, and persistent non-union in 21.4%. Patients with bony union showed significantly higher serum NO level, TGF-β1, VEGF and BMP-2 at 1 month after treatment as compared to patients with persistent non-union. Shockwave-promoted bone healing was associated with significant increases in serum NO level and osteogenic growth factors. The elevations of systemic concentration of NO level and the osteogenic factors may reflect a local stimulation of shockwave in bone healing in long bone non-unions.

Introduction

Non-union of long bone is defined when new bone fails to bridge the fracture gap within 6 months from the initial fracture [1,2]. Many procedures are attempted to prevent the occurrence of non-union including bone grafting, distraction osteogenesis, electrical magnetic field stimulation, low-intensity pulsed ultrasound, gene therapy with BMP-2 and implantation of mesenchymal stem cells [3–10]. Some achieved limited success in selected series, but none showed universal results. Many procedures are invasive and may incur certain risks and complications, and they are costly. Therefore, the development of an effective and safe method of treatment for long bone non-union appears to be very attractive.

Extracorporeal shockwave treatment (ESWT) was shown effective to accelerate bone healing with increased callus formation and to prevent delayed or non-union of long bone fractures [11–17]. Despite the good clinical results, the exact mechanism of shockwave in bone healing remains unknown. Some studies demonstrated shockwave treatment rapidly induces elevation of systemic nitric oxide (NO) level and subsequent increases in systemic osteogenic factors, but not prostaglandin E2 (PGE2) in non-union of long bone [18–21]. Others reported that NO as the mediator in callus formation in fracture healing after mechanical stimulation [22,23]. We hypothesized that local stimulation with ESWT in bone may result in systemic elevations in NO and osteogenic factors. The specific aim of this study was to investigate the effects of shockwave treatment on bone healing and the systemic concentrations of NO level, TGF-β1, VEGF and BMP-2 in long bone non-unions.

Patients and procedures

Institutional Review Board approval was obtained and written informed consent obtained from study subjects. The studies were in compliance with the Declaration of Helsinki ethical principles for medical research involving human subjects. The inclusion criteria comprised of patients with non-unions of diaphyseal fractures of femur and tibia. Non-union was evaluated by history and physical examination, and confirmed by X-rays of the affected bone when the fracture failed to heal in 6 months from the initial treatment. The types of non-union on X-rays included hypertrophic,

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doi:10.1016/j.niox.2009.02.006

Table 1
Patient demographic characteristics.

Number of patients/non-unions	42/42
Age (in years)	
Mean ± SD	34.8 ± 13.6
(Range)	(18–68)
Duration of fracture (in months)	
Mean ± SD	15.02 ± 10.76
(Range)	(6–48)
Gender	
Male	22
Female	20
Anatomical location	
Femoral	28
Tibia	14
Prior operation	
ORIF with IM nailing	31
ORIF with plating	14
ORIF with external fixation	1
Bone grafting	10
Type of non-union	
Atrophic	7
Hypertrophic	35
Length of follow-up (in months)	
Mean ± SD	15.24 ± 7.27
(Range)	(6–24)

atrophic and segmental defect. Patients must be skeletally matured and are competent to sign an informed consent and agree to the follow-up examinations. The exclusion criteria included patients with underlying neoplastic disease or pathological fracture, fracture in the epiphyseal region or active bone infection, patients with fracture near major neurovascular structures such as spine and skull or chest wall, patients with cardiac pacemaker and cardiac arrhythmia, patients receiving immunosuppressive drugs or anticoagulation therapy and pregnancy.

Between February 2005 and December 2006, 42 patients with 42 non-unions of long bone fractures were recruited in this study. There were 22 men and 20 women with an average age of 34.8 ± 13.6 years (range 18–68 years) and an average duration of 15.02 ± 10.76 months (range 6–48 months). All fractures were initially treated with open reduction and internal fixation with intra-medullary nailing in 31 and plating in 10 and external fixation in 1. Ten cases also received bone graft procedure. The sites of non-union were 28 femurs and 14 tibiae. The types of non-union were hypertrophic in 35 and atrophic in 7 based on radiographic appearance. The patient demographic characteristics are summarized in Table 1.

Pre-treatment evaluations included a complete history and physical including the type and frequency of surgery to the fracture, complete blood count including platelet count, coagulation profiles, electrocardiogram and chest X-rays. Ten milliliters of peripheral blood were obtained for measurements of serum NO level and osteogenic markers including VEGF (vessel endothelial growth factor), TGF-β1 (transforming growth factor β1) and BMP-2 (bone morphogenic protein 2); and serum levels of calcium, calcitonin and parathyroid hormone at 1 day before treatment, and at 1 day, 1, 3, 6 and 12 months after treatment. Serum NO level, VEGF, TGF-β1 and BMP-2 were also measured in 16 healthy individuals as the control group.

Shockwave application

The source of shockwaves was from an OssaTron (Sanuwave, Alpharetta, GA). Shockwave treatment was performed with patient on the fracture table under general anesthesia. The focus of the fracture site was verified with C-arm X-rays, and the depth of treatment was determined by raising the height of the table and was confirmed when the two ring markers of the device synchronized under C-arm imaging. Surgical lubrication gel was applied to the skin in direct contact with the shockwave tube. Shockwaves were applied in two planes at 45° to 60° with equal dosage in each plane. Each bone was treated with 6000 impulses of shockwave at 28 kV (equivalent to 0.62 mJ/mm^2 energy flux density) as a single session. A direct contact of shockwave with the metallic devices was avoided. Local swelling, ecchymosis, hematoma, the alignment of the limb, the stability of the fracture and the neurovascular status of the extremity were assessed pre- and post-operatively.

Follow-up examinations were scheduled at 1 day, and 1, 3, 6 and 12 months. Clinical assessments included the intensity of pain based on VAS (visual analogue scale) from 0 to 10 with 0 for no pain and 10 for severe pain at the fracture site, the percent of weight bearing on the affected leg, and the ability to work. Serial radiographs of the affected bone in A-P and lateral views were obtained at each visit to assess the maximal and minimal fracture gaps, the amount of callus formation, and bone healing of the fracture non-union. In case that complete bony union was in question on plain X-rays, three-dimensional computed tomography (3D-CT) was performed to accurately evaluate the healing status of the fracture non-union.

The measurements of serum NO level including nitrite and nitrate were performed using a nitric oxide analyzer (NOA280; Sievers Inc., Denver, USA). The measurements of serum levels of osteogenic markers including TGF-β1, VEGF and BMP-2 were performed using ELISA kits (R & D Systems Inc. Minneapolis, USA) with the specific reagents according to the instructions. The measurements of serum levels of calcium, alkaline phosphatase, calcitonin and parathyroid hormone were performed at our hospital laboratory.

Statistical analysis

The data at different time intervals after treatment were compared with the baseline data before treatment using paired *t* test. The data between patients with bony union and patients with non-union were compared statistically using Mann–Whitney U test. The statistical significance is set at *P*-value <0.05.

Results

The results of clinical assessment are summarized in Table 2. There were significant time-dependent improvements in pain score, weight bearing and work ability after shockwave treatment ($P < 0.05$).

The results of radiographic evaluation are summarized in Table 3. There were progressive improvements in fracture gap, the size of callus and fracture healing, and such changes were time dependent and became significant after 3 months ($P < 0.05$). The rate of bony union was 12% (5 of 42) at 1 month, 43% (18 of 42) at 3 months and 78.6% (33 of 42) at 6 months. At 6 months after ESWT, bony union was noted in 78.6% (33 of 42) and persistent non-union in 21.4% (9 of 42).

The results of serum NO level, TGF-β1, VEGF and BMP-2 are summarized in Table 4. The serum NO level, TGF-β1, VEGF and BMP-2 of the healthy control group are comparable to that of patients with non-union before shockwave treatment. The serum NO level, TGF-β1, VEGF and BMP-2 were significantly higher at 1 month after shockwave treatment as compared to other time courses at 1 day, 1, 3, 6 and 12 months ($P < 0.05$). The serum NO level, TGF-β1, VEGF and BMP-2 were analyzed between patients with bony union and patients with non-union, and the results

Table 2
The results of clinical assessment.

Time	Pre-treatment	1 month	3 months	6 months
Case number	42	42	42	42
VAS (mean ± SD)	3.19 ± 1.55	1.17 ± 1.08	0.45 ± 0.71	0.19 ± 0.4
Range	1–6	0–5	0–2	0–1
P-value		<0.001	<0.001	<0.001
Weight bearing (%) (mean ± SD)	35.35 ± 18.43	47.62 ± 20.81	64.76 ± 22.55	80.0 ± 17.81
Range	10–70	10–80	20–100	50–100
P-value		0.003	<0.001	<0.001
Ability to work (%) (mean ± SD)	25.58 ± 14.19	34.63 ± 18.32	52.2 ± 21.27	76.19 ± 19.87
Range	10–30	10–80	10–100	30–100
P-value		0.007	<0.001	<0.001
Improvement from last examination (%) (mean ± SD)		30.26 ± 13.47	55.00 ± 22.22	79.76 ± 18.93
Range		10–50	20–100	30–100
P-value			<0.001	<0.001

VAS, visual analogue scale from 0 to 10 with 0 for no pain and 10 for severe pain.

Table 3
The results of radiographic evaluation.

Time	Pre-treatment	1 month	3 months	6 months
Case number	42	42	42	42
Maximal fracture gap (mm) (mean ± SD)	3.83 ± 1.34	3.74 ± 1.39	2.63 ± 1.86	1.79 ± 2.15
Range	1.5–7.33	1.2–6.27	0–6.27	0–5.4
P-value		0.381	<0.001	<0.001
Minimal fracture gap (mm) (mean ± SD)	1.98 ± 0.62	1.92 ± 0.68	1.46 ± 0.99	0.9 ± 1.13
Range	0.82–3.94	0.74 –3.94	0–3.94	0–3.94
P-value		0.318	0.002	<0.001
Callus at fracture gap (%) (mean ± SD)		29.88 ± 17.72	57.38 ± 26.14	80.83 ± 24.49
Range		0–75	0–100	25–100
P-value			<0.001	<0.001
Fracture healing by X-ray	0% (0/42)	12%(5/42)	43%(18/42)	78.6%(33/42)
P-value		0.03	<0.001	<0.001

Table 4
Serum NO Level, TGF-β1, VEGF and BMP-2 at different time intervals.

Unit	NO	TGF-β1	VEGF	BMP-2
Normal control (N = 16)				
Mean ± SD	53.2 ± 6.4	42742 ± 4000	383 ± 351	72.4 ± 6.4
P-value[a]	0.06	0.075	0.356	0.381
Pre-treatment (N = 42)				
Mean ± SD	62.2 ± 42.2	49861 ± 10838	334.6 ± 214.5	71.2 ± 16.6
1 day post-treatment (N = 42)				
Mean ± SD	60.6 ± 42.8	46394 ± 14496	359.4 ± 284.3	72.6 ± 17.2
P-value[b]	0.439	0.132	0.345	0.361
1 month post-treatment (N = 42)				
Mean ± SD	92.1 ± 49.2	59166 ± 13547	476.7 ± 306.9	82.1 ± 26.9
P-value[b]	0.003	0.002	0.028	0.018
3 months post-treatment (N = 42)				
Mean ± SD	66.1 ± 43.6	52918 ± 15075	341.1 ± 222.2	69.5 ± 19.6
P-value[b]	0.353	0.189	0.452	0.345
6 months post-treatment (N = 42)				
Mean ± SD	62.5 ± 27.3	48116 ± 13069	269.5 ± 109.8	66.0 ± 23.2
P-value[b]	0.488	0.288	0.074	0.137
12 months post-treatment (N = 42)				
Mean ± SD	50.93 ± 21.98	48609 ± 7228	263 ± 144.9	60.3 ± 28.3
P-value[b]	0.09	0.336	0.099	0.067

Umo/L, micromole/L; Pg/mL, picogram/mL.
[a] Comparison of healthy normal control and patients with non-union.
[b] Comparison of pre-treatment data with the data at 1 day, 1, 3, 6 and 12 months.

are summarized in Table 5. The NO level, TGF-β1, VEGF and BMP-2 of the healthy control group are comparable to patients with non-union before shockwave treatment. After shockwave treatment, patients with bony union showed significantly higher serum NO level, TGF-β1, VEGF and BMP-2 at 1 month as compared to patients with non-union ($P < 0.05$). It appears that shockwave-promoted bone healing is associated with systemic elevations of serum NO level and osteogenesis transduction signals including TGF-β1, VEGF and BMP-2 in long bone non-unions.

The serum levels of calcium, alkaline phosphatase, calcitonin and parathyroid hormone were within normal limits in all cases.

Discussion

Fracture healing is a complex phenomenon involving the growth and differentiation of mesenchymal stem cells, regulation of inflammatory cytokines, synthesis and resorption of extracellular matrix [24–27]. Some studies showed gene expressions of BMP-2, 3, 3B, 4, 6, 7, GDF-5, 7, and BMP antagonists noggin, drm, screlostin, and BAMAI were significantly lower in non-unions compared to standard healing fractures, and concluded that down-regulation in expression of osteogenic BMPs may account for the non-unions of fracture [28]. Other studies demonstrated that areas of newly formed bone had the highest BMP expression that decreased in areas remote from bone formation, and the co-localization of the BMP-2, BMP-4, and BMP-7 proteins with the activated BMP receptors exists in new bone formation [29].

The molecular mechanism of ESWT in bone healing remains unclear. Many studies reported intensive osteochondrogenesis in segmental femoral defects after shockwave treatment, but no shockwave-induced crack or micro-damage was noted [30–32]. Therefore, shockwave-augmented bone formation may be attributed to shockwave-sensitive osteogenesis, rather than damage to the bone architecture. Other studies demonstrated that TGF-β1, BMP-2 and VEGF regulated the mechanical stimulation of fracture healing [33,34]. Recent studies showed that shockwave promotion of fracture healing coincided with increased TGF-β1 and BMP-2

Table 5
Serum NO level, TGF-β1, VEGF and BMP-2 in patients with union and patients with non-union.

NO and osteogenic markers	Normal control ($N = 16$)	Union ($N = 3$)	Non-union ($N = 9$)	P-value[c]
Pre-treatment				
NO (Umol/L)	53.2 ± 6.4	63.6 ± 45.3	57.8 ± 30.5	0.319
P-value[a]		0.12	0.319	
TGF-β1 (Pg/mL)	42742 ± 4000	50722 ± 10807	46275 ± 11175	0.179
P-value[a]		0.075	0.628	
VEGF (Pg/mL)	383 ± 351	347.8 ± 211	289.2 ± 237.3	0.285
P-value[a]		0.361	0.241	
BMP-2 (Pg/mL)	72.4 ± 6.4	72.9 ± 18.0	66.6 ± 6.7	0.074
P-value[a]		0.444	0.079	
1 day post-treatment				
NO (Umol/L)		64.7 ± 47	56.9 ± 19.1	0.264
P-value[b]		0.416	0.494	
TGF-β1 (Pg/mL)		48175 ± 15028	43937 ± 9092	0.107
P-value[b]		0.237	0.349	
VEGF (Pg/mL)		367.6 ± 281	332.7 ± 313.1	0.391
P-value[b]		0.389	0.383	
BMP-2 (Pg/mL)		73.5 ± 18.5	69.1 ± 9.9	0.2
P-value[b]		0.393	0.304	
1 month post-treatment				
NO		99.0 ± 52.3	68.1 ± 26.9	0.017
P-value[b]		0.003	0.228	
TGF-β1		60986 ± 13661	49337 ± 8132	0.034
P-value[b]		0.002	0.306	
VEGF		532.3 ± 318.3	290.7 ± 190.2	0.028
P-value[b]		0.02	0.495	
BMP-2		86.4 ± 29.5	68.8 ± 5.5	0.003
P-value[b]		0.035	0.266	
3 months post-treatment				
NO		67.7 ± 43.9	61.5 ± 44.7	0.361
P-value[b]		0.361	0.408	
TGF-β1		54250 ± 15680	46791 ± 11160	0.09
P-value[b]		0.186	0.468	
VEGF		353.3 ± 194.1	276.2 ± 351.2	0.311
P-value[b]		0.462	0.47	
BMP-2		70.0 ± 21.1	66.5 ± 6.5	0.238
P-value[b]		0.455	0.488	
6 months post-treatment				
NO		64.4 ± 28.1	51.4 ± 21.3	0.168
P-value[b]		0.499	0.36	
TGF-β1		48590 ± 13457	46297 ± 12437	0.309
P-value[b]		0.275	0.498	
VEGF		272.6 ± 113.3	226.6 ± 119.2	0.213
P-value[b]		0.069	0.277	
BMP-2		66.5 ± 24.4	63.5 ± 17.5	0.362
P-value[b]		0.161	0.347	
12 months post-treatment				
NO		50.6 ± 22.5	48.02 ± 13.33	0.399
P-value[b]		0.173	0.358	
TGF-β1		47156 ± 8534	35488 ± 13441	0.134
P-value[b]		0.143	0.153	
VEGF		269.9 ± 159.9	219.4 ± 221.8	0.369
P-value[b]		0.104	0.202	
BMP-2		61.4 ± 28.5	63.83 ± 14.14	0.429
P-value[b]		0.06	0.413	

[a] Comparison of patients with normal control with patients with union and patients with non-union.
[b] Comparison of pre-treatment data with the data at 1 day, 1, 3, 6 and 12 months after treatment.
[c] Comparison of patients with union and patients with non-union.

expressions [30,31] and extracellular signal-regulated kinase (ERK) and P38 kinase in callus [32].

A growing number of studies demonstrated that the increases of systemic osteogenic factors reflecting a local stimulation of bone formation during fracture healing [21,35,36]. Some studies investigated the biological mechanism of ESWT in bone healing at tissue level, and demonstrated that ESWT accelerated fracture healing with the ingrowth of neovascularization and upregulation of angiogenesis and osteogenesis growth factors including eNOS, VEGF, PCNA and BMP-2 [37]. Other studies showed that ESWT triggers the cascade of angiogenic and osteogenic transcription factors (Cbfal/Runx2, HIF-1α and VEGF) in osteoblast cells [38,39]. Many studies showed that acoustic shockwave energy induces nitric oxide (NO) elevation that promotes proliferation and differentiation of human osteoblasts [40]. Nitric oxide, a product from guanidino-nitrogen of L-arginine and dioxygen by three isoforms of nitric oxide synthase (NOS), is a potent regulator for fracture healing in mechanically stimulated bone formation [22,23]. However, no study we are aware of addressed the effects of shockwave on bone healing and the changes in the systemic concentrations of nitric oxide and osteogenic growth factors in long bone non-unions.

The results of the current study showed 79% bony union after ESWT in long bone non-unions that are comparable to treatment with open reduction and bone grafting with no surgical risks [41]. ESWT-promoted bone healing was associated with significant elevations of systemic concentrations of serum NO level, TGF-β1, VEGF and BMP-2 at 1 month after treatment. It appears that shock-wave-promoted bone healing in non-union of long bone was linked to NO modulation and activation of osteogenic growth factors including TGF-β1, VEGF and BMP-2. The releases of systemic NO and osteogenic growth factors after a local application of ESWT to bone appears time dependent with peak levels at 1 month. Therefore, the systemic changes in NO level and osteogenic growth factors may represent a reflection of local stimulation with ESWT in long bone non-unions. It is reasonable to believe that shockwave treatment may provoke NO production, which in turn may activate the mitogenic, osteogenic and angiogenic responses within the bone microenvironment in time fashion.

This study is limited by virtue of the small number of patients creating a relatively low power of statistics. Given the rarity of non-union of long bone fracture, inclusion of a control group in addition to the study group would be neither feasible nor practical in this study. Furthermore, it would be difficult and unpractical to recruit patients with long bone non-union without rendering any form of active treatment. Therefore, no control group was used in the study. The measurements of serum NO level and the osteogenic factors such as TGF-β1, VEGF and BMP-2 were limited to 12 months after treatment under the assumption that bone is less likely to heal further if it fails to heal in 12 months.

In conclusion, ESWT is effective in promoting bone healing in long bone non-unions with a 79% success rate of bony union. Shockwave-promoted bone healing is associated with systemic elevations of serum NO level and osteogenic growth factors including TGF-β1, VEGF and BMP-2. Local shockwave stimulation in bone may reflect the systemic effects of osteogenesis after treatment. Measurements of serum NO level and osteogenic growth factors may be used as the predictors in the assessment of bone healing in long bone non-union.

Acknowledgments

The authors declared that they did not receive any honoraria or consultancy fees in writing this manuscript. No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article. Funds were received in total or partial support for the research or clinical study presented in this article. The funding source was from National Health Research Institute (NHRI-EX96-9423EP).

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