Original Article

Human autologous mesenchymal stem cells with extracorporeal shock wave therapy for nonunion of long bones

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ABSTRACT

Background: Currently, the available treatments for long bone nonunion (LBN) are removing of focus of infection, bone marrow transplantation as well as Ilizarov methods etc. Due to a high percentage of failures, the treatments are complex and debated. To develop an effective method for the treatment of LBN, we explored the use of human autologous bone mesenchymal stems cells (hBMSCs) along with extracorporeal shock wave therapy (ESWT).
Materials and Methods: Sixty three patients of LBN were subjected to ESWT treatment and were divided into hBMSCs transplantation group (Group A, 32 cases) and simple ESWT treatment group (Group B, 31 cases).
Results: The patients were evaluated for 12 months after treatment. In Group A, 14 patients were healed and 13 showed an improvement, with fracture healing rate 84.4%. In Group B, eight patients were healed and 13 showed an improvement, with fracture healing rate 67.7%. The healing rates of the two groups exhibited a significant difference ($P < 0.05$). There was no significant difference for the callus formation after 3 months treatment ($P > 0.05$). However, the callus formation in Group A was significantly higher than that in the Group B after treatment for 6, 9, and 12 months ($P < 0.05$).
Conclusion: Autologous bone mesenchymal stems cell transplantation with ESWT can effectively promote the healing of long bone nonunions.

Key words: *Bone mesenchymal stems cells, extracorporeal shock wave therapy, long bone, nonunion of fracture, transplantation*
MeSH terms: *Stem cell research, bone fracture, bone marrow cell transplantation*

INTRODUCTION

According to statistics, there are around 500 million cases of various types of fractures each year in the United States, and about 5–10% of their patients have delayed or nonunions.[1] Nonunions can be divided into hypertrophic and atrophic nonunion based on biological factors.[2] In traditional treatment methods (such as bone grafting and internal fixation surgery), with longer time and higher medical costs, surgical trauma can result in insufficiently derived autologous bone and allogeneic bone exclusion of the body after surgery. Therefore, clinicians have continuously searched for minimally invasive, more economically reliable, and effective methods of treatment. Various bone growth inducing factors and the physical means to promote bone healing have been introduced with molecular biology. Surgical treatment is no longer the only feasible method with the development of biophysical techniques.

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How to cite this article: Zhai L, Ma XL, Jiang C, Zhang B, Liu ST, Xing GY. Human autologous mesenchymal stem cells with extracorporeal shock wave therapy for nonunion of long bones. Indian J Orthop 2016;50:543-50.

Extracorporeal shock wave therapy (ESWT) is a noninvasive method with a significant effect on tissue injuries. Recently, ESWT has been applied for the treatment of nonunions with a success rate of 60–90%.[3] Moreover, ESWT is a choice of treatment for nonunions and delays bone healing because of its minimally invasiveness and simplicity.[3] In the United States and Europe, the law permits clinical orthopedic surgeons to use ESWT for the treatment of calcific supraspinatus tendinitis, lateral epicondylitis, plantar fasciitis, bone nonunion, and other stubborn muscle diseases.[4-6]

Bone mesenchymal stems cells (BMSCs) can be transdifferentiated in the differentiation potential of cells present in a pair of stages. Nuttall *et al.*[7,8] found that the expression of osteocalcin (OCN) can completely induce bone cells into fat cells with BMSCs as the source. Fully differentiated mature hypertrophic chondrocytes can differentiate into bone cells. Meanwhile, the reticular fiber cells in the body can be transformed into fat cells. Fat cells can be cultured *in vitro* to differentiate into bone cells and reverse the formation of real bone tissue, indicating that BMSCs have strong plasticity. The use of BMSCs combined with ESWT for the treatment of nonunion remains to be elucidated. Studies have demonstrated that the grafting of human mesenchymal stem cells (MSCs) is an effective and safe method for the treatment of tibial diaphyseal nonunion,[9-12] and ESWT is especially effective for hypertrophic nonunions of long bone.[13-18] This study was aimed to combine the advantages of different therapies, further improve the healing rate and shorten the treatment time of nonunion.

MATERIALS AND METHODS

63 patients of nonunion long bones treated by autologous BMSCs transplantation ESWT were included in the study.

The protocols in this study were approved by the Medical Ethic Committee of the General Hospital as well as Medical University committee for stem cell research.

The diagnostic criteria for the nonunions were as follows: At least 6 months after the fracture but has not been healed yet and no further tendency of healing for 3 months is apparent.[19] The subjects selected for this study were at least 8 months after the fracture. The age ranged between 20 and 50 years.

The exclusion criteria were as follows: (1) Patients have coagulation disorder or are taking anticoagulant drugs, (2) local tumor patients, (3) local thrombosis, (4) infected nonunion, (5) a large section of nonunion defects, with defects >1.5 cm, (6) 18 years of age or immature bone

development, (7) patients with severe cardiovascular and cerebrovascular disease, (8) patients with severe malnutrition, (9) pregnancy, (10) local inflammation, (11) heart disease, and (12) the fracture ends are active. To this end, 63 out of 80 subjects were selected for this study between 2004 and 2010. The 63 patients were randomly divided into the combined transplantation of autologous BMSC–ESWT treatment group (A Group, 32 cases) and simple ESWT treatment group (B Group, 31 cases).

Group A consisted of 18 male and 14 female patients with a mean age of 39.6 years (range 23-50 years). The bones included were as follows: Femoral shaft fractures ($n = 10$), tibial shaft fractures ($n = 15$), humeral shaft fractures ($n = 5$) and ulnar shaft fractures ($n = 2$). The average duration to diagnose nonunions was 13.4 months (range 9–20 months).

Group B consisted of 18 male and 13 female patients with an average age of 38.1 years (range 20-49 years). The bones included were as follows: Femoral shaft fractures ($n = 11$), tibial shaft fractures ($n = 13$), humeral shaft fractures ($n = 5$), ulnar shaft fracture ($n = 1$) and radial shaft fracture ($n = 1$). The average duration to diagnose nonunions was 12.9 months (range 9-19 months).

In Group A, three cases of atrophic nonunion, two cases of distal tibial 1/3 (one case with plate and screw fixation, one case of fixed external fixation after the operation), one case of the humerus (plate screw internal fixation), and the rest were hypertrophic nonunion. In Group B, there were also three cases of atrophic nonunion, two cases of distal tibial 1/3 (both external fixation after the operation), one case of middle (plate screw internal fixation), and the rest was hypertrophic nonunion.

The differences in gender, age, nonunion type, healing time, location, and specific information between the two groups were not statistically significant ($P > 0.05$).

Extracorporeal shock wave therapy treatment
The power at the focus point was defined as the energy flux density (EFD) per impulse and measured in millijoules per square millimeter. EFD was not defined for some devices, and the energy level of shock waves was specified in kilovolts. High-energy shock wave therapy was performed in the present study with an electrohydraulic MFL 5000 Lithotriptor (Dornier Medizintechnik GmbH, Wessling, Germany). The number of shock waves varied with the location and length of the nonunion (approximately 1000/cm). A mean of 2900 shock waves with an average voltage of 23 kV was applied.

The exact EFD has not been evaluated for the MFL 5000. However, compared with the newer Epos Fluoro

Lithotriptor, the approximations that were impacted by the Dornier Company confirmed that high-energy level of the shock waves was applied with more than 0.7 mJ/mm^2 EFD. During the treatment, the upper operating and leg voltage were 10–14 kV and 11–13 kV, respectively, with 2000 frequencies of each impact. To the patients with internal fixations (22 cases in Group A and 19 patients in Group B), we used shockwave from the side of no internal fixations. Each treatment had an interval of 3 days, and the treatment frequency was 4 times for the upper limbs and 5 times for the lower limbs [Figure 1].

Purification and identification of bone mesenchymal stem cells

The patients in Group A were characterized through laboratory tests to exclude blood and other organ diseases. The patients also signed informed consents. Under sterile conditions, the bone marrow puncture was done and bone marrow aspiration was taken from the posterior superior iliac spine, following 4.5 mL heparin saline (200 U/mL) anticoagulant injection. The clean bench within the Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum culture medium was diluted after slowly adding the cell suspension along the wall with a centrifuge. The Ficoll-Paque reagent (1.077 g/mL, GE Healthcare Grade) was utilized for the concentration of bone marrow cells. The medium in the centrifuge tubes was separated into a centrifugal radius of 12 cm at 2000 r/min. After gradient centrifugation for 20 min, the middle of the interface cell suspension was drawn to obtain the monocyte hBMSC. The monocyte hBMSC was further purified by washing through DMEM medium and centrifugation (centrifugal radius of 12 cm, 1500 r/min). The count of concentrated cells was adjusted to 1×10^6 cells/mL with a flow cytometry. The mononuclear cells and BMSCs uniformly expressed CD44, CD90, and CD105 but did not express CD34, CD45, and CD14. The cell purity was more than 95%, indicating that the isolated surface exhibited highly uniform characteristics of hBMSCs. The hBMSC were continuously passaged until P3 (the 3rd generation) before autologous transplantation. The direct inoculation, Tachypleus amebocyte lysate, as well as membrane filtration methods were processed to test the sterility of the transplant material. The procedure was performed in a surgical setting.

Autologous transplantation of human autologous bone mesenchymal stems cells

The affected drape area was strictly sterilized, and the three-dimensional fluoroscopic monitoring was performed under the guidance of fluoroscopy nonunion ends, which were gently peeled with a needle to create accommodation space. The needle stylet was pulled out, and the catheter body was purified with 2–3 mL of mononuclear cell suspension (density $>1 \times 10^7$ cells/mL) before slowly injecting it into the nonunion site (one injection to complete). The cell suspension was evenly distributed in the fracture and the pressure bandage in the puncture was applied with a sterile dressing [Figure 2].

The criteria for the clinical healing of the fracture were as follows: (1) No local tenderness and vertical percussion pain; (2) no local abnormal mobility (automatic or passive); (3) X-ray showed a blurred fracture line, in which
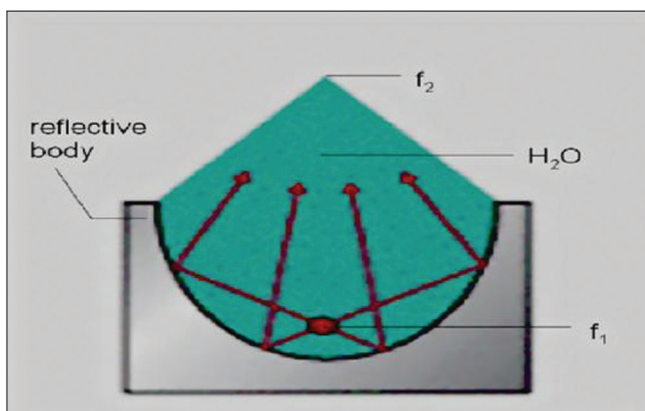

Figure 1: Schematic diagram showing the liquid-electric shock wave source: F1 is the first focus (source occurred); F2 is the second focus (the impact point of nonunion)
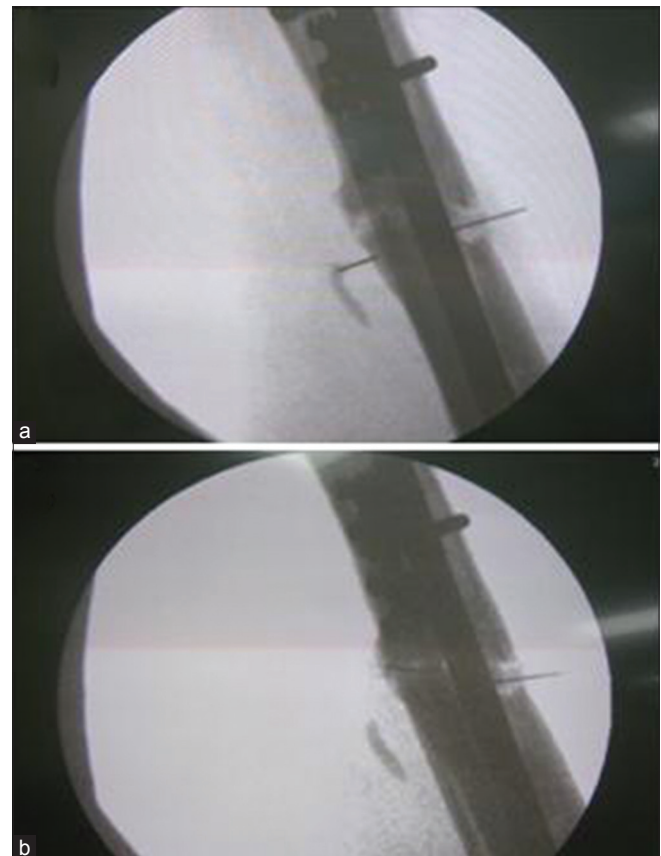

Figure 2: Puncture in the X-ray orientation (pictures taken by c-arm) showing (a) Anterior view; (b) lateral view

a continuity of the bone crust was observed through the fracture line; and (4). After removing, the external fixation of the injured limb, the upper limb, forward flexion and holding with 1 kg weight for 1 min, and the lower limbs can walk without crutches continuously for 3 min and <30 steps.

The efficacy assessment was as follows: Healing if the fracture was healed within 12 months according to criteria 1–4 and the fracture line disappeared; improved fracture healing if the results were in line with criteria 1 and 2 within 12 months, the callus formation was clearly visible, and the fracture line was still present; invalid if the fracture line is still present, no callus formation and the results did not meet the criteria within 12 months.

Healing rate = (number of cases of clinical basic healing/
              treatment of patients with a total number)
              × 100%.

Callus formation

Callus formation was obtained with Perkins' formula, given by $2\pi r 1 (r^2 - r^1) 1$, where r^1 is the radius of bone, r^2 is the radius of bone after the callus, and 1 the length of the callus. PowerNet Viewer software, Finland,[19] was used to obtain the values of r^1, r^2, and 1, and then the values were calculated with the formula.

Statistical analysis

Measurement data were expressed as a mean ± standard deviation, and an independent sample *t*-test was used to compare the two groups. The test was used to compare rates Chi-square with a test level of $\alpha = 0.05$.

RESULTS

The two groups of patients underwent followup tests for 12 months to 18 months, with an average of 17.1 months. After 3 months treatment, there was no significant callus formation in both groups. After 6 months treatment, the X-ray films showed that twenty patients in Group A and 17 patients in Group B had varying degrees of callus formation. After 9 months treatment, 23 patients in Group A and 19 patients in Group B had varying degrees of callus formation. After 12 months treatment, 14 patients were healed, 13 patients showed an improvement and a fracture heating rate of 84.4% were observed in Group A. Moreover, eight patients were healed, 13 patients showed an improvement and a fracture healing rate of 67.7% were observed in Group B. Both groups had significant differences in healing rate ($P < 0.05$) [Table 1 and Figures 3-6].

There were five patients in Group A and 10 in Group B with treatment failures, and they were particularly involved

Table 1: The quantity of callus formation after treatment with ESWT group and the group of ESWT combined with autograft of hMSCs (cm³, $\bar{x}\pm s$)

Group	n	3 months	6 months	9 months	12 months
A	32	3.611±0.770	6.896±3.129	9.654±2.997	11.908±3.000
B	31	3.402±1.081	5.702±2.5489	6.100±2.923	8.410±1.098
P		>0.05	<0.05	<0.05	<0.01

ESWT=Extracorporeal shock wave therapy, hMSCs=Human mesenchymal stem cells

in the atrophic type of pseudarthrosis and congenital bone disorders such as fibrous dysplasia and osteogenesis imperfecta excluding pathological fractures. This skewed distribution may due to the better treatment of Group A.

DISCUSSION

In 1976, Friedenstein *et al.* first reported a small portion of bone marrow samples of adherent cells in culture that were able to be differentiated into bone cells, cartilage cells, fat cells, and muscle cells. These cells were called as MSCs, which maintained their multidirectional differentiation potential. MSCs with an injury in the ischemic tissue exhibited homing characteristics. Studies have shown that autologous red bone marrow injections can promote the healing of fractures.[20-22]

Haupt *et al.*[23] performed extracorporeal shock wave (ESW) treatment on ureteral stones, and found an increase in partial hip bone cell activity that confirmed the potential of ESW to promote bone formation. Wang *et al.*[24] investigated the function of ESWT in the bone progenitor cell colony-forming units (CFU-O), bone marrow stromal cell CFU (CFU-S), and whole blood cell CFU (CFU-M) of rat femur proliferation and differentiation. The results showed that 0.16 mJ/mm² and 500 frequency ESW partial fracture fragments could yield CFU-O and CFU-S concentration, accelerated proliferation, and differentiation of BMSCs. CFU-M did not have this effect.

Some scholars have found that ESWT applications depend on time and energy and have cumulative effects.[25,26] Wang *et al.*[27] found that by using ESW intervention, BMSCs can induce the production of transforming growth factor-β1 5 min after the occurrence of membrane potential hyperpolarization, 30 min after the cells were activated by the Ras system and 6 h after BMSCs promote differentiation to osteoblasts. The following are the observation 2 days after the cells began to proliferate. ALP increased 6 days after the cells were proliferated into the bone within the specificity of the nuclear transcription factor, and the expression of Col-I increased. The formation of the bone section was ultimately induced 12 days after increasing of OCN synthesis, indicating that ESW can promote BMSCs for osteoblast growth and differentiation of precursor cells. Haake *et al.*[28]

Figure 3: X-ray anteroposterior and lateral view of leg bones in a 32 years old female showing (a) hypertrophic nonunion tibia following closed reduction and internal fixation (b) 9 months after human autologous mesenchymal stem cells with extracorporal shock wave therapy

Figure 4: X-ray right elbow joint, anteroposterior view in a 44 years male showing (a) hypertrophic nonunion supracondylar fracture which was treated conservatively (b) 6 months after treatment with human autologous mesenchymal stem cells and extracorporeal shock wave therapy showing union

Figure 5: X-ray left leg bones anteroposterior and lateral views in a 55 years old male, with open fracture, treated in external fixator showing (a) nonunion tibia (b) 4 months after treatment with human autologous mesenchymal stem cells and extracorporeal shock wave therapy showing union

investigated the function of the different energy densities of ESW in cultured human bone marrow cells and found that low-energy ESW caused changes in cell morphology and number. Moreover, the increase in energy density and frequency of bone marrow cells drastically affected the hole size, number, and fragmentation. ESWT could also cause the proliferation of cultured bone marrow cells. Martini *et al.*[29] investigated the effects of different energy levels and a number of shocks of ESW on human osteoblast-like cells through the cell culture experiments.

In this study, after 3 months treatment, there was no significant callus formation in both groups. After 6 months

treatment, the X-ray films showed that twenty patients in Group A and 17 patients in Group B had varying degrees of callus formation. After 9 months treatment, 23 patients in Group A and 19 patients in Group B had varying degrees of callus formation. After 12 months treatment, 14 patients were healed, 13 patients showed an improvement and a fracture healing rate of 84.4% were observed in Group A. There was a significant difference in healing rate between the two groups. This result coincided with the "time-dependent," "cumulative effect," and "energy dependence" theory. In addition to the mentioned effects, ESW-vibration micro-environment with osteoblast-like cells is also important. Wet bone has
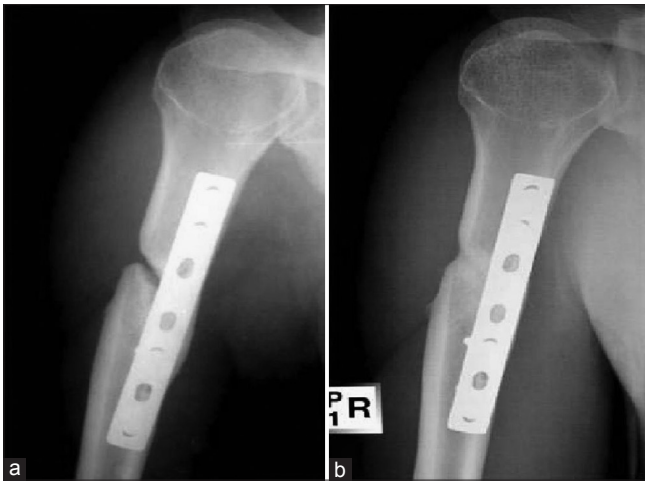
Figure 6: X-ray (R) arm anteroposterior view in a 24 years male showing (a) hypertrophic nonunion with plate in situ (b) 4 months after treatment with human autologous mesenchymal stem cells and extracorporeal shock wave therapy showing union

a galvanic potential and piezoelectric potential, and ESW produced by electromagnetic vibrations can promote bone regeneration and increase growth. Moreover, hormone and growth factors support extracellular matrix synthesis.[30] ESWT intervention by the end of the hardening of bone fractures caused by trauma responds to the new area of the extended inflammation. The stimulated vascular response to these stimuli can cause the growth of many new blood capillaries and can bring a large number of cytokines and growth factors on cell proliferation and differentiation.

ESWT and the average nonunion fracture hastened union and stopped local pain after conservative treatment, without adverse effects on the local soft tissues and bones. No systemic contraindication was observed, therefore it was considered as a feasible ESWT treatment. However, the local inflammatory response of patients with serious cardiovascular and cerebrovascular diseases must be carefully managed. The application of this procedure in cases with surgical treatment of bone, atrophy, and infected nonunion for atrophic nonunion was limited because ESWT treatment could not induce new bone formation. ESWT treatment may cause the spread of infection in infected nonunion, therefore, it was not conducive to bone healing. In large segments of defective nonunion, bone defects were >2 cm.[31-33]

However, experimental evidence[34] has shown that ESW has a stimulating effect on the cells, and the shock wave energy stimulation inhibits the cells. Cell inhibition is probably the result of high-energy density after the shock wave treatment, which then causes a significant local subcutaneous bleeding and swelling that are detrimental to the healing of nonunion. In the treatment of nonunion, surgery with sophisticated fixation devices like intramedullary nails, ilizarov external fixator, unilateral fixator, and autologous bone grafting still represented the gold standard. Basbozkurt *et al*.[35] reported a bony consolidation of humerus pseudarthroses had a success rate of about 80% with the ilizarov external fixator. The treatment of femoral nonunions with intramedullary nails by Pihlajamäki *et al*.[36] had a success rate of about 90%. In tibial nonunions, percutaneous grafting with bone marrow autologous alone had a success rate of about 90%, while with expandable intramedullary nailing and autologous bone grafting had a success rate of above 90%.[37-41] In the study, a fracture healing rate of 84.4% was observed in Group A, and a fracture healing rate of 67.7% was observed in Group B. Compared with the effects, the healing rate of A Group with the gold standard (autograft) seems to be inferior to some, but in consideration of the therapy of minimal invasion, fewer side effects, the treatment we think is satisfied.

CONCLUSIONS

The treatment of nonunion, with surgical therapy and autologous bone grafting is gold standard. At present, high-energy ESWT is still regarded as experimental. Adding hBMSCs clearly improves the efficiency of ESWT, which provide a new prospective for the future study.

Financial support and sponsorship

Conflicts of interest
There are no conflicts of interest.

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