Effect of extracorporeal shock wave on proliferation, differentiation, adhesion and migration of rat osteoblasts *in vitro*★

Huang Zhong-lian, Hu Jun, Yu Meng-lei, Lu Zhi-jun

Abstract

BACKGROUND: Extracorporeal shock wave therapy is indicated as an effective method for treatment of delayed fracture healing or nonunion. Osteoblasts plays an important role in this process.
OBJECTIVE: To investigate the function of osteoblasts in the process of extracorporeal shock wave promoting fractures healing, and to provide theoretical support for improving shock wave therapy on fracture healing.
METHODS: Primary cultured ostsoblasts were isolated from newborn SD rat calvaria and randomly divided into two groups, shock wave and control. Treated by different energies of extracorporeal shock wave, cells were incubated onto 96-well culture plate. An optimal dose of extracorporeal shock wave was selected according to survival and proliferation of osteoblasts. The osteoblasts treated by optimal energy of extracorporeal shock wave were cultured and harvested for the analysis of alkaline phosphatase by calcium cobolt stain, cell survival by CCK-8 Kit, alkaline phosphatase expression by AKP kit, mineralized nodules by Alizarin red staining, integrin β_1 and β_1 mRNA expressions by flow cytometry and RT-PCR, cell migration by wound healing assay.
RESULTS AND CONCLUSION: The optimal energy of extracorporeal shock wave treating primary cultured osteoblasts was 10 kV (500 impulses). Following extracorporeal shock wave therapy, the cell proliferation, alkaline phosphatase activity, cell mineralization, rates of cell adhesion, as well as β_1 integrin and its mRNA expressions were increased as compared with those in control group ($P < 0.01$). Further distance of cell migration was found in extracorporeal shock wave group ($P < 0.05$). The results showed that the optimal energy of extracorporeal shock wave could promote the proliferation, differentiation, adhesion and migration of osteoblasts *in vitro*, and β_1 integrin may play an important role in the process of cell adhesion and migration.

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INTRODUCTION

Bone defect and nonunion following fracture are challenging problems facing orthopedic surgeons. The traditional method, autologous bone grafting, is limited by local complications and finite supply of autologous bone grafts. In addition, autologous bone grafting surgery is an invasive process and is not indicated for high risk patients. Bone fracture healing is a complex process with constant bone remodeling and new bone formation, involving multiple cell types including osteoblasts and osteoclasts, numerous cellular factors such as bone morphogenetic proteins and transforming growth factor, as well as extracellular matrix. Osteoblasts as bone-forming cells can differentiate into osteocytes after migrating to specific sites after bone fracture. In the meanwhile, they secrete bone matrixes and promote their mineralization, thus promoting formation of bone tissues. Differentiation and migration of osteoblasts is a key to bone morphogenesis, remodeling and healing.

Shock wave causes a transient pressure disturbance with a sudden rise from the ambient pressure to its maximal pressure. It has been applied for lithotripsy and osteoporosis[1-2]. It has also been used for treating fracture and chronic degenerative diseases such as musculoskeletal disorders[3]. Shock wave could activate osteoblasts and possess potential of bone promoting function, thus capable of promoting healing of bone fracture[4]. However, the mechanisms underlying shock wave promoting bone fracture healing are still not fully elucidated. In addition, no previous report focuses on the effect of shock wave on the adherence and migration of osteoblasts. This study was aimed to isolate and identify osteoblasts from rat bone tissues, and sought to investigate the effects of shock wave on the proliferation, differentiation and migration of osteoblasts *in vitro*.

MATERIALS AND METHODS

Design

A cytological and biological *in vitro* experiment.

Time and setting

This study was performed at the Laboratory of Molecular Biology in the First Affiliated Hospital of Shantou University Medical College between August 2008 and April 2009.

Materials

Three-day old Sprague-Dawley (SD) rats, of either gender and of SPF grade, were provided by the Experimental Animal Center of Shantou University Medical College. All experimental procedures were in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[5].

Methods

Isolation of osteoblasts from rats

Three-day old SD rats were sacrificed by decapitation and immersed in 75% alcohol for 5 minutes. The skull was then prepared under sterile conditions. After removal of the soft tissue and the periosteum, bone was cut into small pieces at

1 mm ×1 mm in size placed in fetal bovine serum, which then grew in 25-cm^2 tissue flask in Dulbecco's modified eagle's medium containing 10% fetal bovine serum at 37 ℃ in a humidified atmosphere containing 5% CO_2. Cell growth was observed daily under an inverted microscope (Nikon, Japan) and half of the medium was replenished when the cells showed radial growth from the bone surface. Thereafter, the medium was replaced every three days, when the cells covered 90% of the flask bottom, they were digested with 0.25% trypsin and passaged. Osteoblasts were purified by the attachment method as previously described[6].

Identification of osteoblasts

The purified osteoblasts were identified using alkaline phosphatase assays and by calcified nodule staining[7]. Briefly, for alkaline phosphatase assay with the modified calcium-cobalt staining method, osteoblasts at the 4th passage were inoculated in 6-well plates at a density of 1×10^8/L. When the cells were 50% confluent, the media was removed and the cells were washed twice with phosphate buffered saline (PBS), which were then fixed in 95% alcohol at 4 ℃ for 15 minutes. After washed with distilled water twice, the cells were incubated with calcium-cobalt staining substrates at 37 ℃ for 6 hours. Following washed with distilled water, the cells were stained in 2% cobalt nitrate solution for 5 minutes, and after rinsed with tap water, cells were put into 1 % ammonium sulfide solution for 2 minutes. The cells were then rinsed with tap water for 3 minutes and let dry naturally. Ten fields were subsequently randomly selected at 100 × magnification and the cells were counted in each field with cells containing dark brown particles considered osteoblasts and the positive rate of osteoblasts was calculated.

For staining of calcified nodule with alizarin red, after osteoblasts were in inoculated for 3 weeks, when multiple transparent calcified nodules were observed, the media was removed. The nodules were washed twice with PBS and then fixed in 95% alcohol at 4 ℃ for 15 minutes. Following washed twice with distilled water, 0.1% alizarin red-Tris-HCl staining solution (pH 8.3) was added, after incubation at 37 ℃ for 30 minutes, the nodules were rinsed with distilled water and let naturally dry. The calcified nodules were then observed and photographed at 100 × magnification.

Treatment of osteoblasts with shock wave

Osteoblasts from the 4th passage were digested with 0.25% trypsin and rendered into single cell suspension. The cells were then placed into 15-mL sterile polystyrene tubes at a concentration of 1×10^9/L. The cells were treated with shock wave as described previously by Wang *et al*[8]. Briefly, the tubes were exposed to a single shock wave treatment of 0, 5, 10, 15 or 20 kV for 500 impulses using Huikaing type IV shock wave equipment (Huikang, Shengzhen, China).

Cell survival, proliferation and differentiation assays

Osteoblasts at the 4th passage that were treated with shock wave at 5, 10, 15 or 20 kV for 500 impulses were stained by trypan blue to assess the survival of the osteoblasts. For cellular proliferation assays, the treated osteoblasts or control cells were added to 96-well plates at 1×10^4/well and were allowed to grow for 24 and 48 hours. The growth of these cells was determined using the CCK-8 kit as instructed by the manufacturer (Dojindo Laboratories, Kumamoto, Japan). Absorbance at 450 nm was read with a microplate reader (Bio-Rad, USA).

For determination of osteoblastic differentiation, cells treated with shock wave or control cells were inoculated for 2 weeks and the media was saved and replenished every 3 days. Alkaline phosphatase content was determined using a commercial alkaline phosphatase kit as instructed by the manufacturer (Jiancheng Biotechnological Co., Nanjing, China). Absorbance at 520 nm was read and alkaline phosphatase content (μkat/L) was calculated. Furthermore, cells treated with shock wave or control cells were inoculated for 3 weeks and were then stained with alizarin red as described above. The calcified nodules were photographed at 100 × magnification and red-stained nodules with a clear margin and a short diameter greater than 20 mm were selected and analyzed using Image Pro Plus6.0 software (Media Cybernetics Co, USA). For the deposition of calcified salts in the extracellular matrix in the shock wave treatment group and the control group[9].

Determination of adherence rate of osteoblasts

The attachment by osteoblasts to the wall of culture flask was observed every 2 hours under an inverted microscope. The cells were digested and rendered into single cell suspensions. The cells (1×10^4/well) were inoculated into 96-well plates and each group was inoculated in sextuplets. The cells were removed for counting at 2, 4, 6, 8 and 10 hours after inoculation, and the adherence rate for the shock wave group and the control group was calculated.

Determination of integrin β_1 expression on osteoblasts

For determination of the expression of integrin β_1 on osteoblast, the attached cells were digested and centrifuged at 1 000 r/min for 5 minutes. The precipitates were suspended in PBS and centrifuged at 1 000 r/min for 5 minutes. The cellular precipitates were then suspended in 100 L PBS at a concentration of 5×10^8/L. Anti-FITC-β_1 integrin antibody was then added, after incubation at 4 ℃ for 45 minutes, the mixture was centrifuged at 1 000 r/min for 5 minutes and the precipitates were re-suspended in PBS. The mean fluorescent intensity of integrin β_1 was determined by flow cytometry. For measurement of integrin β_1 mRNA level, 24 hours after shock wave treatment, total cellular RNA was extracted using the Trizol reagents as instructed by the manufacturer (Invitrogen, Carlsbad, CA) and 1 μg total RNA was reverse transcribed into cDNA using a commercial reverse transcription kit (Tiangen, Beijing, China).

The sequences of primers for PCR were as follows:

GAPDH:
upper strand: 5'- ACC ACA GTC CAT GCC ATC AC-3'
lower strand: 5'-CCA CCA CCC TGT TGC TGT A-3'
product size: 452 bp

Integrin β_1:
upper strand: 5'-GGA GGA ATG TAA CAC GAC TGC-3'
lower strand: 5'-CAG ATG AAC TGA AGG ACC ACC-3'
product size: 585 bp

The PCR was run at 94 ℃ for 30 seconds, 55 ℃ for 30 seconds and 68 ℃ for 60 seconds for a total of 33 cycles. The PCR products were resolved by 2% agarose gel electrophoresis and visualized under a UV transilluminator after staining with GoldView and photographed. The band intensity was determined using the software Quantity one and was normalized against GAPDH mRNA.

Determination of osteoblast migration

Wound healing test was performed to measure osteoblast migration. Briefly, adherent cells were digested with trypsin and rendered into single-cell suspensions. The cells were inoculated at 1×10^4/well in 6-well plates. When the cells became fully confluent, a scar was made along a straight line using a 200-μL pipette and the floating cells were rinsed with PBS. The scar was marked in the plate and the cells were inoculated in serum-free media. Six points were photographed at 12 and 24 hours after the scar was made under a microscope at 40 × magnification, and the mean migration rate was determined and analyzed using the Image Pro Plus 6.0 software.

Main outcome measurement

Alkaline phosphatase staining of osteoblasts was detected by calcium cobalt staining method, cell proliferation by CCK-8 Kit, the expression of alkaline phosphatase by AKP Kit, mineralized nodule by Alizarin Red staining, the expression of β_1 integrin by flow cytometry and their mRNA expressions by RT-PCR, the migration of osteoblasts by wound healing test.

Design, enforcement and evaluation

The first and second authors designed this study. All authors performed experimental procedures. The third and fourth authors evaluated experimental data. All authors received professional trainings. No blind method was used for the evaluation.

Statistical analysis

The data were expressed as Mean ± SD, and analyzed using SPSS 13.0 software (SPSS, Chicage, IL, USA). One-way analysis of variance was used for comparison among multiple groups, and paired-samples *t* test was used for comparing the mean between the shock wave treatment group and the control group. A level of $P < 0.05$ was considered statistically significant.

RESULTS

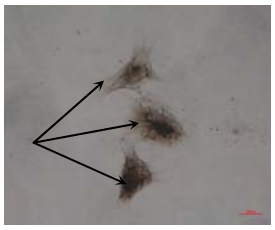
Shock wave induced a dose-dependent effect on the survival and growth of osteoblasts

The osteoblasts isolated from bones in the skull of rats were observed. Inverted microscopy revealed that these osteoblasts were spindle, triangular or polygonal shaped. Calcium cobalt staining further showed that the isolated cells became adherent, most of these cells were stained dark brown and expressed potent alkaline phosphatase activity (Figure 1a). After three weeks of culture, (88.9±3.2)% of the isolated cells were identified to be osteoblasts. Alizarin red staining revealed the presence of multiple red nodules, which were various in sizes (Figure 1b), further confirming that these cultured cells were osteoblasts.

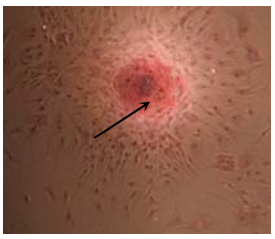
Primary osteoblasts were processed to shock wave treatment at 5, 10, 15, or 20 kV for 500 impulses. At 24 hours after treatment, osteoblasts receiving 5 and 10 kV of shock wave showed no statistically significant difference in the number of viable cells as revealed by trypan blue staining ($P > 0.05$), while osteoblasts receiving 15 or 20 kV of shock wave showed significantly reduced survival compared with control osteoblasts ($P < 0.01$) (Figure 2).

Cell proliferation assay showed that, 24 hours after shock wave treatment, there was no statistically significant difference between osteoblasts receiving 5 or 10 kV and the control cells ($P > 0.05$), while shock wave at 15 or 20 kV markedly reduced the proliferation of osteoblasts compared with controls ($P < 0.01$) (Figure 3a).

Furthermore, at 48 hours, shock wave at 5 kV induced no statistically significant difference in the proliferation of osteoblasts compared with controls ($P > 0.05$), while shock wave at 10 kV caused a markedly increase in the proliferation of osteoblasts compared with controls ($P < 0.05$). Further increase of the energy level of shock wave to 15 or 20 kV caused a significant reduction in the proliferation of osteoblasts ($P < 0.05$) (Figure 3b). These findings indicate that shock wave caused a dose-dependent effect on the survival and growth of osteoblasts and 10 kV for 500 impulses was an optimal level for shock wave treatment for osteoblasts.

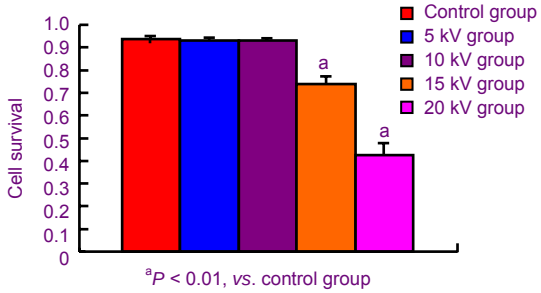


a: Alkaline phosphatase staining of osteoblasts by calcium cobalt method (× 400)



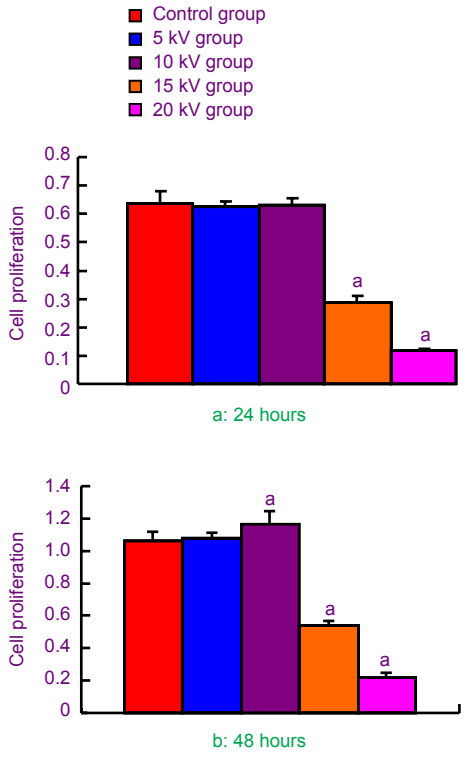
b: Alizarin red staining of mineralized nodule (× 100)

Figure 1 Identification of osteoblasts



[a]$P < 0.01$, *vs.* control group

Figure 2 Cell survival of osteoblasts after shock wave treatment

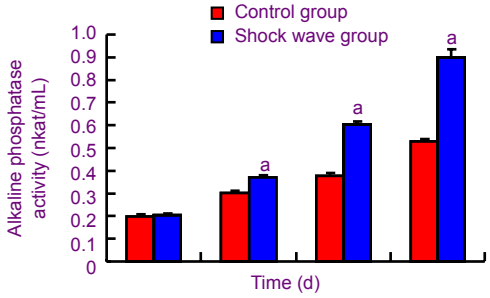


[a]$P < 0.05$, *vs.* control group

Figure 3 Cell proliferation of osteoblasts after shock wave treatment

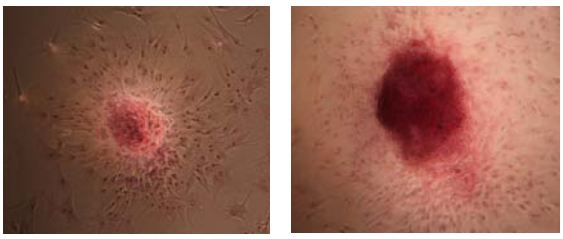
Shock wave stimulated the secretion of alkaline phosphatase from osteoblasts and increased the size of calcified nodules

We further measured secreted alkaline phosphatase activity in control cells and osteoblasts receiving 10 kV of shock wave treatment. There was no statistically significant difference in the secreted alkaline phosphatase activity at day 3 after shock wave treatment ($P > 0.05$). However, 6−12 days after shock wave treatment, a statistically significant increase in the secreted alkaline phosphatase activity was observed in the shock wave treatment group compared with the control group ($P < 0.05$) (Figure 4). Alizarin red staining of calcified nodules showed that the size of calcified nodules in the shock wave treatment group (2.66±0.33) was markedly larger than that of controls (0.97±0.29) ($P < 0.01$) (Figure 5), suggesting that shock wave treatment was associated with a higher level of calcified salt deposition.



[a]$P < 0.05$, *vs.* control group

Figure 4 The expression of alkaline phosphatase in shock wave group and control group



a: Control group b: Shock wave group

Figure 5 Alizarin Red staining of mineralized nodule in shock wave group and control group (× 100)

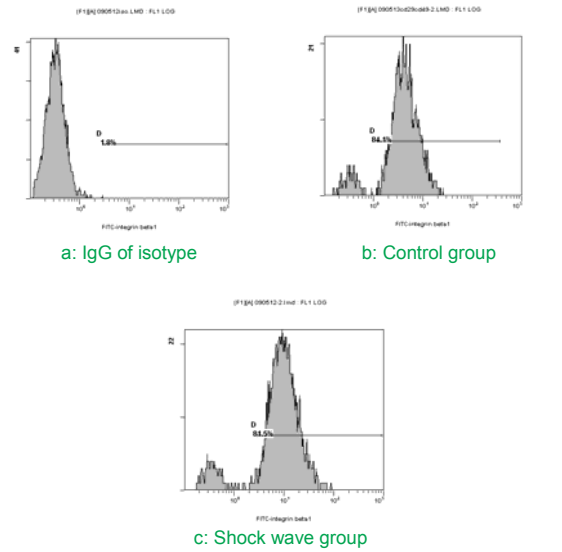
Shock wave stimulated the expression of integrin β1 and the adherence of osteoblasts

There was a markedly increased adherence of osteoblasts to the wall of tissue flask 2−6 hours after shock wave treatment (10 kV, 500 impulses) compared with controls ($P < 0.05$), indicating that shock wave stimulated the adherence capacity of osteoblasts.

At 24 hours after shock wave treatment, a marked increase was seen in the expression of integrin β_1 in osteoblasts treated with shock wave (10.27±0.30) compared with controls (6.88±0.31) ($P < 0.01$).

Shock wave treatment of osteoblasts caused a significant increase in the mRNA level of integrin β_1 (0.81±0.03) compared with controls (0.50±0.04) (Figure 6).

The above findings suggested that shock wave increased the expression of integrin β at both the translational and transcriptional level.



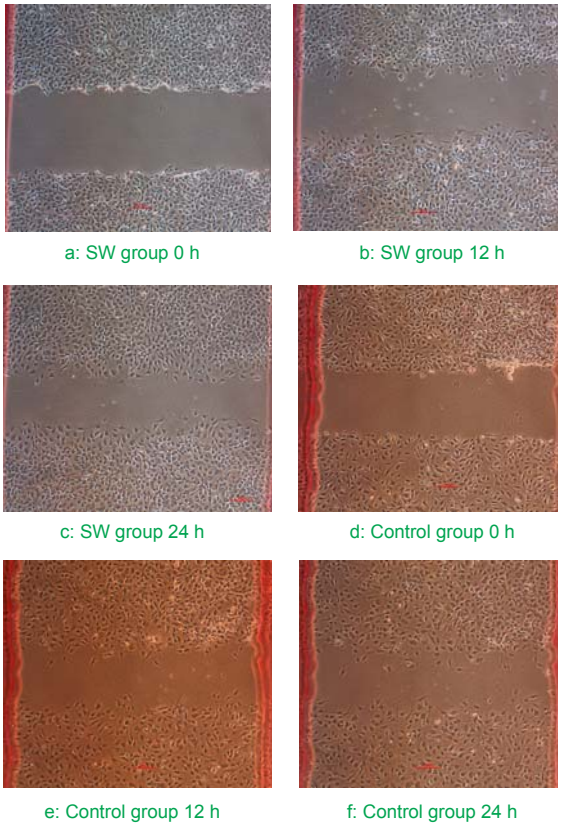
a: IgG of isotype b: Control group

c: Shock wave group

Figure 6 The expression of β_1 integrin as shown by flow cytometry

Shock wave promoted the migration of osteoblasts

The mean migration of osteoblasts in the short wave treatment group at 12 and 24 hours after wound formation (0.90±0.10, 1.73±0.17) was greater than that of controls (0.74±0.13, 0.99±0.11) ($P < 0.05$, $P < 0.01$) (Figure 7), suggesting that shock wave could promote the migration of osteoblasts.



a: SW group 0 h b: SW group 12 h

c: SW group 24 h d: Control group 0 h

e: Control group 12 h f: Control group 24 h

Figure 7 The images of cell migration in shock wave (SW) group and control group (× 100)

DISCUSSION

Osteoblasts derive mostly from pluripotent bone marrow stromal mesenchymal cells. They are involved in osteogenesis and bone morphogenesis, and are responsible for the synthesis and secretion of bone matrix, thus providing a scaffold for bone growth. Osteoblasts promote new bone formation and play an important role in bone remodeling, and transforming growth factor-β_1 has been shown to stimulate the differentiation and proliferation of osteoblasts during bone healing after fracture[10]. Alkaline phosphatase is an early marker and calcified nodules are considered a late marker for osteoblast differentiation[11-12]. In this study, osteoblasts were isolated from bone tissues by culturing harvested bone tissue blocks. Both alkaline phosphatase activity assays and calcium-cobalt staining revealed potent production by the cultured osteoblasts of alkaline phosphatase. Alizarin red staining further showed the presence of calcified nodules, suggesting that these cells were capable of depositing calcified salts *in vitro*. In addition, our finding that (88.9±3.2)% of the cultured cells were osteoblasts suggests that the purified cells were a highly enriched population of osteoblasts.

Shock wave has been shown to promote bone healing following fracture in animals, and could be effective as a physical therapy for patients with nonunion fracture[4,13-17]. A shock wave generates a transient pressure disturbance in the targeted tissue and propagates rapidly in three dimensions. It causes a cavitation effect consequent to the negative phase of the wave propagation. However, the mechanisms whereby shock wave promotes bone healing after fracture have not been fully elucidated. Extracorporeal shock wave was shown to up-regulate the expression of bone morphogenesis proteins-2, -3, -4, -7 in the bone marrow stromal mesenchymal cells, immature chondroblasts and osteoblasts at the fracture site. They could stimulate the differentiation and proliferation of osteoblasts and chondroblasts[18]. It also increased the recruitment of bone marrow stromal mesenchymal cells to the bone defect and markedly up-regulated the expression of transforming growth factor-β1 and vascular endothelial growth factor-α[19], these two cytokines that can induce the differentiation and proliferation of mesenchymal cells to osteoprogenitors. A shock wave treatment of 10 kV for 500 impulses applied to cultured osteoblasts, may increase proliferation of osteoblasts and enlarge calcified nodules, suggesting that shock wave stimulated the differentiation and proliferation of osteoblasts. The increased deposition of calcified salts in the extracellular matrix as evidenced by the enlarged calcified nodules indicates that shock wave also promotes the differentiation of osteoblasts at the late stage of osteoblast differentiation.

Integrins are membrane proteins that are mainly involved in mediating the adherence between cells, between cells and extracellular matrix, through binding to ligands in the extracellular matrix, it activates intracellular signaling pathways that promote cellular growth, differentiation, adherence and migration[20]. The expression of integrins varies in osteoblasts at different stages of osteocyte development, the β subunit is the major integrin receptor for important ligands in the extracellular matrix such as collagen, fibronectin, and laminin[21-22]. We isolated osteoblasts from bone tissues by culturing harvested bone tissue blocks, without the use of lengthy enzymatic digestion, thus avoiding compromising the integrity of receptors or antigens on the membrane[23]. We examined the expression of integrin β_1 on osteoblasts and found that shock wave significantly increased both the protein and mRNA levels of integrin β_1. Integrin β_1 is known to mediate the adherence of osteoblasts to the extracellular matrix, and to regulate the synthesis of the extracellular matrix[24]. Results found that the shock wave-induced upregulation of integrin β_1 was associated with increased adherence of osteoblasts to the surface of culture flasks and enhanced migration of these cells after wound formation, suggesting that the promotion of bone healing after fracture by shock wave could also be attributed to increased migration and adherence of osteoblasts in the fracture site.

In conclusion, shock wave stimulates the migration and adherence of osteoblasts, which may be a mechanism whereby shock wave promotes bone healing following fracture.

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体外冲击波对大鼠成骨细胞增殖、分化、黏附及迁移的影响*

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摘要

背景：体外冲击波是治疗骨折延迟愈合或不愈合的一种有效方法，成骨细胞在此过程中发挥着重要的作用。

目的：研究成骨细胞在体外冲击波促进骨折愈合过程中的作用，为提高冲击波治疗效果提供理论支持。

方法：将原代培养的 SD 大鼠成骨细胞随机分成冲击波组和对照组，应用不同能量的冲击波进行处理后接种于 96 孔培养板，根据细胞存活率和细胞增殖情况确定适宜的冲击波能量值。钙钴法染色观察成骨细胞碱性磷酸酶，CCK-8 试剂盒检测细胞存活，AKP 试剂盒检测 AKP 表达，茜素红染色观察矿化结节，流式细胞仪检测整合素 β_1 及 RT-PCR 检测整合素 β_1 mRNA 表达，伤口愈合试验观察成骨细胞的迁移率。

结果与结论：冲击波处理体外原代培养成骨细胞的适宜能量为 10 kV(500 脉冲)，冲击波组细胞增殖速度快，细胞分泌碱性磷酸酶水平高，矿化结节面积大，细胞黏附率高，整合素 β_1 及其 mRNA 的表达均高于对照组($P < 0.01$)，且冲击波组细胞迁移的平均距离大于对照组($P < 0.05$)，提示适宜能量冲击波可促进成骨细胞的增殖、分化、黏附及迁移，同时，整合素 β_1 在细胞黏附及迁移过程中可能扮演了重要的角色。

关键词：冲击波；成骨细胞；增殖；分化；黏附；迁移；整合素；骨组织工程
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*利益冲突：*课题未涉及任何厂家及相关雇主或其他经济组织直接或间接的经济或利益的赞助。

*课题的意义：*体外冲击波治疗非侵袭性、并发症少，随着研究的深入，未来可作为手术的替代疗法应用于骨折不愈合和延迟愈合的治疗之中。

*课题评估的“金标准”：*CCK-8 是检测增殖能力的最新通用方法，碱性磷酸酶检测及矿化结节茜素红染色是成骨细胞分化检测的公认指标，细胞划痕试验是检测细胞迁移的经典设计。

*设计或课题的偏倚与不足：*尚未对发生机制可能涉及的具体作用通路未进入深入分析，有待后续研究。

*提供临床借鉴的价值：*实验为临床更加广泛应该冲击波治疗提供治疗能量值参考及理论支持。