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The dose–effect relationship in extracorporeal shock wave therapy: the optimal parameter for extracorporeal shock wave therapy

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ABSTRACT

Background: Extracorporeal shock wave therapy (ESWT) has been demonstrated to have the angiogenic effect on ischemic tissue. We hypothesize that ESWT exerts the proangiogenesis effect with an energy density–dependent mode on the target cells.

Materials and methods: Endothelial progenitor cells (EPCs) of rats were obtained by cultivation of bone marrow–derived mononuclear cells. EPCs were divided into five groups of different energy densities, and each group was furthermore subdivided into four groups of different shock numbers. Thus, there were 20 subgroups in total. The expressions of angiogenic factors, apoptotic factors, inflammation mediators, and chemotactic factors were examined, and the proliferation activity was measured after ESWT.

Results: When EPCs were treated with low-energy (0.04–0.13 mJ/mm^2) shock wave, the expressions of *endothelial nitric oxide synthase, angiopoietin (Ang) 1, Ang-2, and B-cell lymphoma 2* increased and those of *interleukin 6, fibroblast growth factor 2, C-X-C chemokine receptor type 4, vascular endothelial growth factor a, Bcl-2-associated X protein, and caspase 3* decreased. *stromal cell-derived factor 1* changed without statistical significance. When cells were treated with high-energy (0.16 mJ/mm^2) shock wave, most of the expressions of cytokines declined except the apoptotic factors and *fibroblast growth factor 2*, and cells lead to apoptosis. The proliferation activity and the ratio of *Ang-1/Ang-2* reached their peak values, when cells were treated with ESWT with the intensity ranging from 0.10–0.13 mJ/mm^2 and shock number ranging from 200–300 impulses. Meanwhile, a minimal value of the ratio of *Bax/Bcl-2* was observed.

Conclusions: There is a dose–effect relationship in ESWT. The shock intensity ranging from 0.10–0.13 mJ/mm^2 and shock number ranging from 200–300 impulses were the optimal parameters for ESWT to treat cells *in vitro*.

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1. Introduction

Extracorporeal shock wave is a longitudinal acoustic wave that propagates through water or soft tissue as ultrasound does [1]. Extracorporeal shock wave was first used in lithotripsy. Studies have demonstrated that low-energy extracorporeal shock wave therapy (ESWT) is effective for nonunion of fractures, tendinitis, and aseptic necrosis of bone in orthopedics [2]. It is also used to treat ischemia disease such as angina and ischemia heart failure in cardiology [3,4]. We have reported that ESWT could improve the healing of diabetic wound and the survival of ischemia skin flap in rats [5,6]. ESWT has been considered as a promising noninvasive angiogenic strategy [7].

However, the mechanism of ESWT is still not clear [8]. Some researchers have reported that ESWT could improve the expression of angiogenic factors [6,9] in stem cells and protect cells from apoptosis [9]. But the dose–effect relationship between the intensity and the biological effects of shock wave is poorly investigated.

Endothelial progenitor cells (EPCs) are the progenitors of endothelial cells with robust angiogenic activity [10]. Researches revealed that EPCs circulate from bone marrow to incorporate into and contribute to postnatal physiological and pathologic neovascularization [11,12]. Furthermore, EPCs have recently been suggested to represent a promising cellular tool [13,14]. Therefore, in the present study, EPCs were used as the targets of ESWT.

We hypothesize that the ESWT exerts its proangiogenesis effect with an energy density–dependent mode on the target cells. Accordingly, the present study aimed to reveal the dose–effect relationship in ESWT, and determine the optimized parameter (including energy flux density and shocks) for ESWT to treat cells cultured *in vitro*.

2. Material and methods

2.1. EPCs cultivation

All animal procedures were approved by the Laboratory Animal Ethics Committee of Shanghai Jiaotong University and conformed to the National Guidelines for Care and Use of Laboratory Animals. A total of 12 male Sprague-Dawley rats (Laboratory Animal Research Center, Shanghai), each weighing 250–300 g, were used in the experiment. According to the method described in the literature [15], bone marrow tissue was aspirated from bone marrow cavity of femurs and tibias of rats. The bone marrow tissue was flushed out several times with low glucose Dulbecco's Modified Eagle Medium (DMEM) (Hyclone, Beijing, China) supplemented with 20% fetal bovine serum (Hyclone). The bone marrow cell suspension was filtered through a 40-μm strainer, and the fraction of mononuclear cells was separated by Histopaque (1.083 g/mL; Sigma-Aldrich, St. Louis, MO) density–gradient centrifugation. *Ex vivo* expansion of EPCs was performed as previously described [16,17]. Briefly, these mononuclear cells were plated on rat fibronectin (Calbiochem, Merck, Darmstadt, Germany)–coated culture dishes and maintained in an Endothelial Basal Medium-2 (EBM-2) (Lonza, Basel, Switzerland) supplemented with Endothelial Cell Growth Media 2 (EGM-2) MV SingleQuots (Lonza, Basel, Switzerland) (including 5% fetal bovine serum, hydrocortisone, vascular endothelial growth factor [VEGF], human fibroblast growth factor b, human epidermal growth factor, insulin-like growth factor 1, and ascorbic acid). After 4 d, nonadherent cells were removed by washing, new media were added, and the culture maintained through 16 d, and plenty of cells ($\sim 1.0 \times 10^6$) were found to grow on the plate. After all the operations, the animals were returned to animal center and kept as usual.

2.2. Identification of EPCs

To identify the population of EPCs cultivated, the cells were trypsinized, washed twice with phosphate-buffered saline (PBS), and immunostained for 30 min on ice with the following antibodies: polyclonal sheep antibody against rat CD34 (R&D, Minneapolis, MN), DyLight 488–conjugated monoclonal antibody against kinase insert domain receptor (KDR) (Novus, Littleton, CO), fluorescein isothiocyanate (FITC)-conjugated antibody against Von Willebrand factor (vWF) (Abcam, Cambridge, MA). Cells labeled with nonfluorescence-conjugated antibodies were then incubated with Alexa Fluor 488–conjugated antibodies specifically against sheep immunoglobulin G (Jackson, West Grove, PA). Isotype-identical antibodies (immunoglobulin G) served as controls. Flow cytometric analyses were performed using fluorescence–activated cell sorter (Navios, Bechman Coulter, Brea, CA). DiI-Ac-Low-density lipoprotein (LDL) (Molecular Probes; Invitrogen, Eugene, OR) uptake test and FITC-Ulex europaeus (UEA) (Sigma, St. Louis, MO) binding test were also performed. Cells staining positively for both markers were considered to be differentiating EPCs as reported previously [17,18].

2.3. Group division and shock wave treatment

EPCs of third passage were used in the experiment. Cell suspension was collected in a centrifuge tube by trypsinization, and the concentration was adjusted to 1.0×10^5/mL. EPCs were divided into five groups with energy flux densities: 0.04, 0.07, 0.10, 0.13, and 0.16 mJ/mm^2. Each group was subdivided into four subgroups with shock numbers: 140, 200, 300, and 500 impulses. Thus, there were 20 subgroups in total. ESWT with different parameters was applied on cell suspension in a centrifuge tube accordingly. The centrifuge tubes were smeared with ultrasound transmission gel (Pharmaceutical Innovations Inc, NJ) as contact medium between the tube and the ESW apparatus (Orthospec, Medispec Ltd, Yehud, Israel). After shock wave treatment, the EPCs were reseeded on plates to continue cultivation separately according to different subgroups. After 48 h, Methyl Thiazolyl Tetrazolium (MTT) assay and real-time quantitative polymerase chain reaction (PCR) were performed to examine the proliferation activity and expression of cytokines.

2.4. MTT assay for proliferation activity

Treated with shock waves of different intensity, 2000 cells of third passage per well were seeded in fibronectin–coated

96-well plates for cultivation at 37°C. After 48 h, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 g/L) (Sigma) was added to each well, and then the cells were cultured for 4 h. After the removal of the supernatant, 150 μL of dimethyl sulfoxide (Gibco, Invitrogen, Grand Island, NY) was added to each well. After shaking for 10 min at room temperature, cell proliferation was assessed by measuring the absorbance at 492 nm with a microplate reader (Synergy HT, Bio-Tek, Winooski, VT).

2.5. *Real-time quantitative PCR for angiogenic, apoptotic, inflammation, and chemotactic factors*

Treated with shock waves of different intensity, EPCs were cultured at 37°C in 5% CO_2 for 48 h. Total RNA from cell samples was isolated by using Trizol reagent (Invitrogen, Grand Island, NY) according to the manufacturer's protocol. A total of 1 μg of RNA sample was reverse transcribed with oligo dt15 by avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI). Real-time quantitative PCR (7500 sequence detection system, ABI, Invitrogen, Grand Island, NY) amplifications were performed by using the power SYBR green PCR master mix reagent (Invitrogen), and primer pairs used are listed in Table. β-Actin messenger RNA (mRNA) from the same sample was used as an internal control. The following cytokines were examined: angiogenic factors: *endothelial nitric oxide synthase (eNOS)*, *vascular endothelial growth factor a (VEGFa)*, *angiopoietin (Ang)* 1, and *Ang-2*; apoptotic factors: *Bcl-2-associated X protein (Bax)*, *Bcl-2-associated X protein (Bax)*, and *caspase 3*; inflammation mediators: *interleukin 6 (IL-6)* and *fibroblast growth factor 2 (FGF-2)*; chemotactic factors: *C-X-C chemokine receptor type 4 (CXCR4)* and *stromal cell-derived factor 1 (SDF-1)*.

2.6. *Statistical analysis*

All experiments were performed in triplicate and repeated three times. Quantitative data are expressed as mean ± standard deviation. Statistical analysis was adequately performed by an analysis of variance followed by the Tukey multiple comparison procedure. Statistical analysis was performed using SPSS for Windows ver. 13.0 (SPSS, Inc, Chicago, IL). A probability value of $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. *Cell cultivation and identification*

About 4–6 d after seeding, spindle-shaped cells appeared. Gradually, spindle-shaped cells disappeared and colonies of cobble stone–like cells emerged after 7–9 d. After cultivating for 16 d, cobble stone–like cells were found attached to the culture dish. Flow cytometric analysis showed strong expression of CD34, KDR, and vWF, along with the ability of being double stained by DiI-Ac-LDL and FITC-UEA, indicating the phenotype of an EPC (Fig. 1).

3.2. *Viability measurement by MTT assay*

After culturing for 48 h, cells treated with shock waves of different intensity showed different proliferation activity (Fig. 2). Comparing among groups with same shock number (Fig. 2A), the absorbance value rose with the increase of energy flux density and reached peak value when energy density ranged from 0.07–0.13 mJ/mm^2, and then decreased below normal at 0.16 mJ/mm^2.

Table – Primers used in real-time quantitative PCR.

Gene name	Genbank_ID	Primer sequence	Product length (bp)
β-actin	NM_031144.3	Forward: TCCTGGCCTCACTGTCCACCT Reverse: CGCAGCTCAGTAACAGTCCGCC	115
Bcl-2	NM_016993.1	Forward: TCCTTCCAGCCTGAGAGCAAC Reverse: CGACGAGAGAAGTCATCCCC	164
Bax	NM_017059.2	Forward: ATGGCCTCCTTTCCTACTTCGG Reverse: AGAAAAGACACAGTCCAAGGCA	136
eNOS	NM_021838.2	Forward: ATCAGCAACGCTACCACGAG Reverse: CTGTCGCTCCTGCAAAGAAA	101
VEGFa	NM_031836.2	Forward: GAACCAGACCTCTCACCGGAA Reverse: GACCCAAAGTGCTCCTCGAAG	136
Caspase 3	NM_012922.2	Forward: CTGGACTGCGGTATTGAGAC Reverse: CCGGGTGCGGTAGAGTAAGC	104
FGF-2	NM_019305.2	Forward: GCGACCCACACGTCAAACTA Reverse: AGCAGCCGTCCATCTTCCT	109
Ang-1	NM_053546.1	Forward: CCCGTCTTGAAATCCAACTGC Reverse: TCTGACGAGTAACCAAGCCTT	210
Ang-2	NM_134454.1	Forward: ACTGACGCACATCACTTAGGC Reverse: ACACTGTAGAAACGTGCTCCGA	243
CXCL12	NM_022177.3	Forward: TGCATCAGTGACGGTAAGCCA Reverse: ATCCACTTTAATTTCGGGTCAA	188
CXCR4	NM_022205.3	Forward: TCCTGCCCACCATCTATTTTATC Reverse: ATGATATGCACAGCCTTACAT	226
IL-6	NM_012589.2	Forward: TCCTACCCCAACTTCCAATGCTC Reverse: TTGGATGGTCTTGGTCCTTAGCC	79

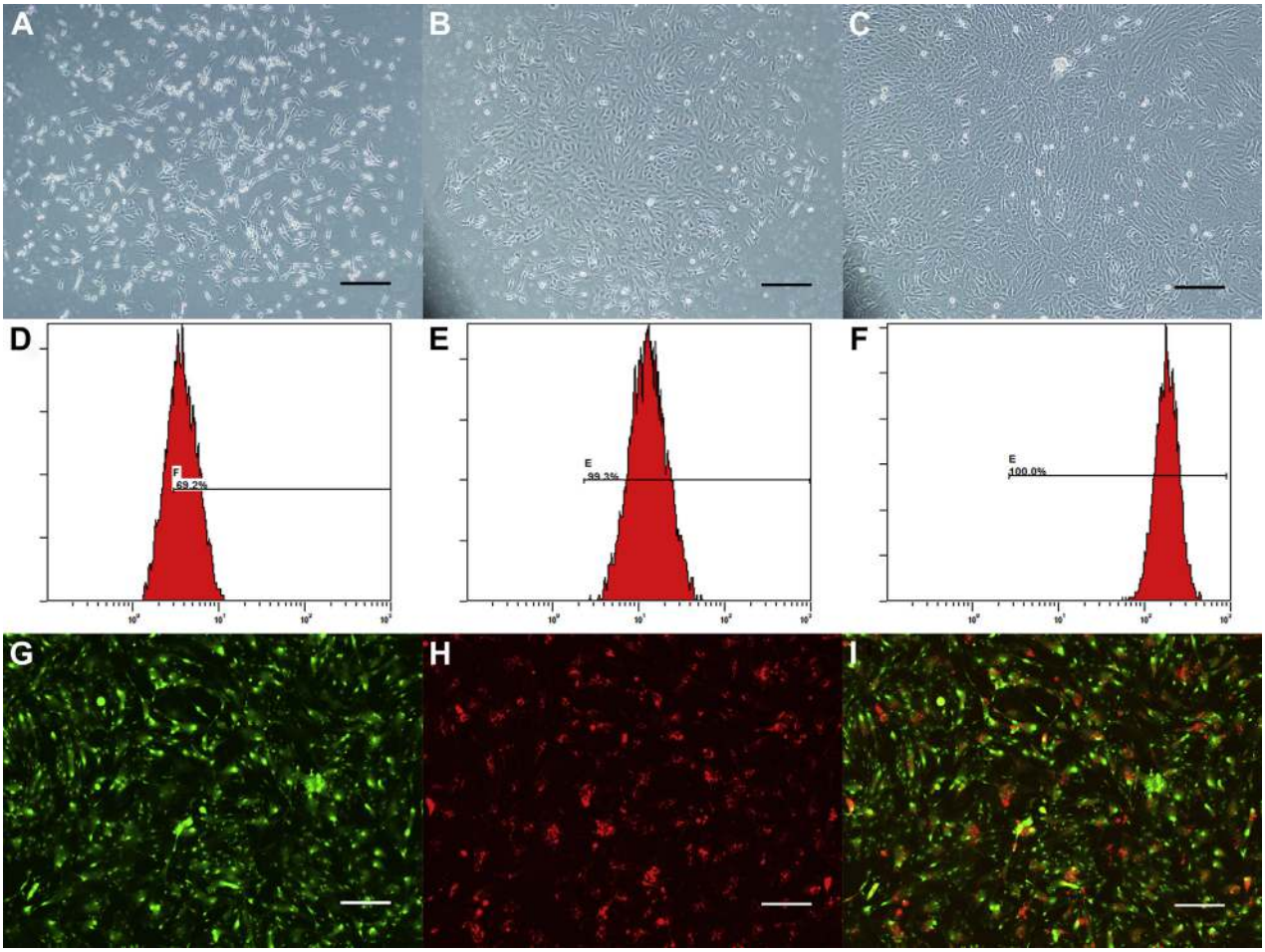

Fig. 1 – The cultivation and identification of EPCs: (A) After 4–6 d of seeding. (B) After 7–9 d of seeding. (C) After cultivating for 16 d. (D–F) Flow cytometric analysis showed strong expression of vWF (D), CD34 (E), and KDR (F). (G) Uptake of DiI-Ac-LDL by EPCs. (H) Binding of FITC-UEA-1 by EPCs. (I) Double stained by DiI-Ac-LDL and FITC-UEA, indicating the phenotype of endothelial progenitor cell. Scale bars represent 200 μm. (Color version of figure is available online.)

Among groups with same energy flux density (Fig. 2B), the impact of shock energy on cell proliferation ability showed a same trend like the impact of different impulses. The absorbance value increased with the increase of shock impulses and reached the peak value when energy parameter ranged from 200–300 impulses, and then decreased at 500 impulses.

Combining the two results, the strongest viability appeared in 0.10 mJ/mm^2 with 300 impulses subgroup when compared with other subgroups ($P \leq 0.05$). A total of

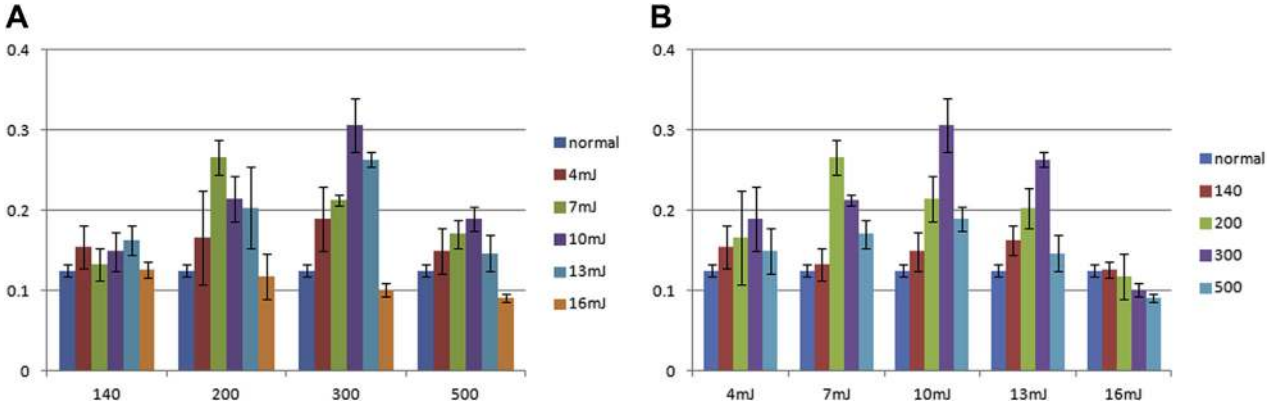

Fig. 2 – Viability measurement by MTT assay. (A) Effect of different shock impulses on proliferation. (B) Effect of shock waves of different intensities on proliferation, the strongest viability appeared in the subgroup of 0.10 mJ/mm^2 with 300 impulses ($P \leq 0.05$). (Color version of figure is available online.)

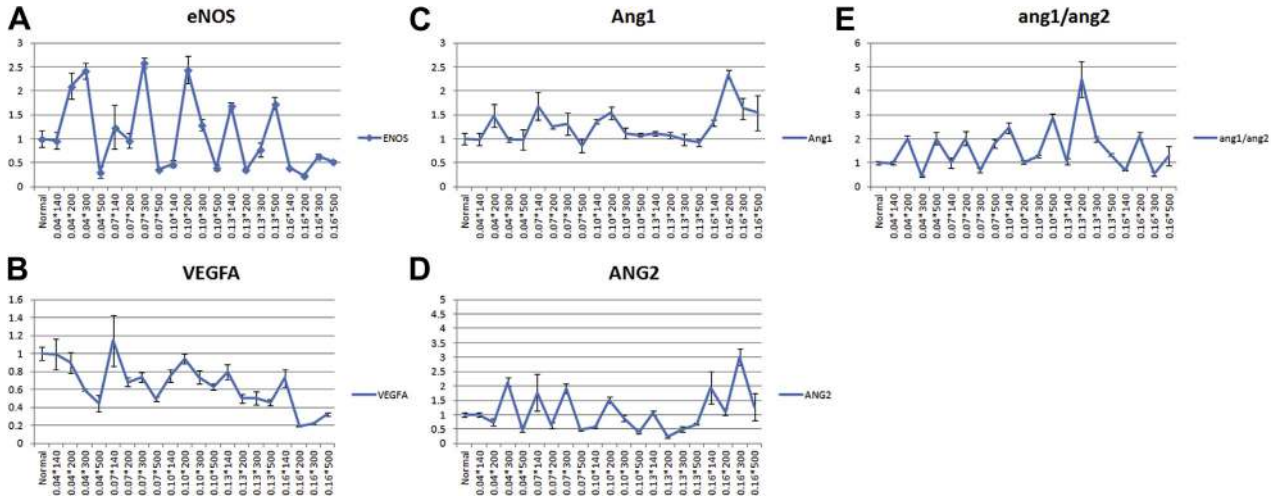

Fig. 3 – Measurement of the expression of angiogenic factor by real-time quantitative PCR. Effect of different parameters on (A) eNOS, (B) VEGFa, (C) Ang-1, and (D) Ang-2. (E) The ratio of Ang-1 and Ang-2, the value reached the peak value in 0.13 mJ/mm^2 with 200 impulses subgroup. (Color version of figure is available online.)

0.13 mJ/mm^2 with 300 impulses subgroup and 0.07 mJ/mm^2 with 200 impulses subgroup also showed stronger viability than the rest of subgroups ($P \leq 0.05$), although the two subgroups did not have statistical significance with each other ($P > 0.05$).

3.3. *Measurement of angiogenic factor expressions by real-time quantitative PCR*

ESWT showed different effect on the expression of different cytokines (Fig. 3). Under the stimulation by shock wave with low intensity, *eNOS* expressions in most of the subgroups increased above normal level and the highest value appeared in 0.04 mJ/mm^2 with 300 impulses subgroup ($P \leq 0.05$), the second and the third were in 0.07 mJ/mm^2 with 300 impulses subgroup and 0.10 mJ/mm^2 with 200 impulses subgroup, although the fluctuation was large among groups with same energy flux density. With the increase of the shock wave intensity, the peak value of each energy group decreased gradually. In high energy group (0.16 mJ/mm^2), there was no value higher than the control group (Fig. 3A).

However, the expression of *VEGFa* did not increase after shock wave treatment, and decreased gradually with the increase of shock wave energy and shocks (Fig. 3B).

After cells were treated with ESWT, the expression of *Ang-1* (Fig. 3C) and *Ang-2* (Fig. 3D) increased above the normal value obviously, although the absolute value decreased gradually with the increase of shock wave intensity. But in the groups with high energy density (0.16 mJ/mm^2), the expression of two cytokines increased again and reached its peak value. The trends of the two cytokines were almost same. However, the ratio of *Ang-1/Ang-2* increased with the increase of the intensity of shock wave, not like the trends of *Ang-1* and *Ang-2*. When the intensity was 0.13 mJ/mm^2 with 200 impulses, the ratio reached its peak value with statistical significance ($P \leq 0.05$), then the value decreased to near normal in the high energy group (0.16 mJ/mm^2) (Fig. 3E).

3.4. *Measurement of inflammation mediators by real-time quantitative PCR*

Expression of two representative inflammation mediators, *IL-6* and *FGF-2*, decreased obviously when the cells were treated with low-intensity ESWT. However, under the impact of shock wave with high energy flux density (0.16 mJ/mm^2), the trend of *FGF-2* expression reversed and increased

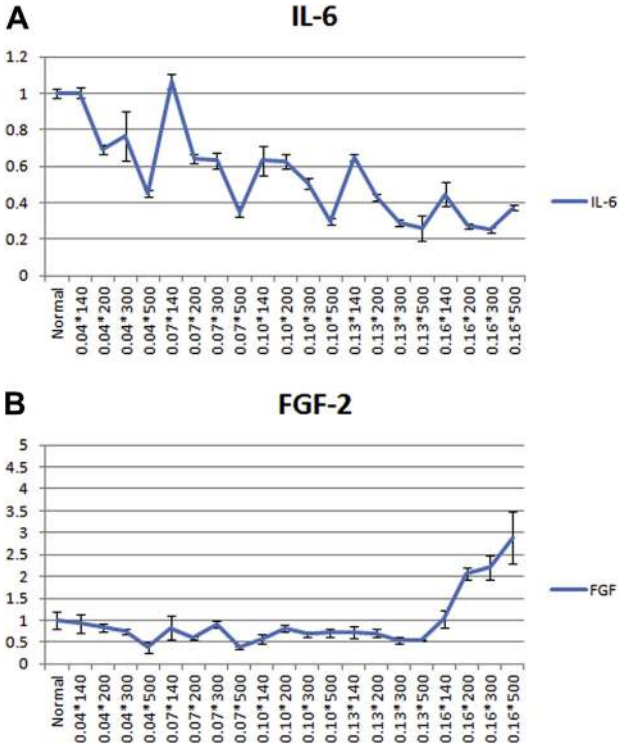

Fig. 4 – Measurement of inflammation mediators by real-time quantitative PCR. Effect of different parameters on (A) IL-6 and (B) FGF-2. (Color version of figure is available online.)

significantly (Fig. 4B). But the expression of *IL-6* still decreased and reached the lowest level (Fig. 4A). The two changes were with statistical significance ($P \leq 0.05$).

3.5. *Detection of the apoptosis indicators by real-time quantitative PCR*

Expression of *caspase 3* in EPCs decreased after ESWT. With the increase of shock wave energy, the level of *caspase 3* increased again, but did not reach the normal (Fig. 5A). However, the situation of *Bax* and *Bcl-2* was different from *caspase 3*. When treated with low-energy (<0.13 mJ/mm^2) shock wave, most of *Bax* expressions (Fig. 5B) were lower than the normal level but most of *BCL-2* expressions (Fig. 5C) were higher than the normal level. With the increase of the shock wave energy, the level of *BAX* increased by some extent, and increased significantly under high energy stimulation (0.16 mJ/mm^2). Under the same condition, *BCL-2* also increased by large scale, but the increase was less than *Bax*. So the ratio of *Bax/BCL-2*, which related to cell apoptosis, was at the lowest level in the range from 0.10–0.13 mJ/mm^2 with statistical significance ($P \leq 0.05$), indicating the antiapoptotic effect of ESWT with low energy. Its peak value was in the group at 0.16 mJ/mm^2, indicating the proapoptotic effect of ESWT with high energy (Fig. 5D).

3.6. *Detection of expression of chemotactic factors by real-time quantitative PCR*

The two chemotactic factors showed different appearances. *SDF-1* did not change a lot with the increase of energy flux density and shocks, although there was fluctuation within the curve. Most of the comparisons among these values did not have significant difference ($P > 0.05$) (Fig. 6). But *CXCR4* decreased sharply when treated with shock wave. And the lowest value was in 0.16 mJ/mm^2 with 200 impulses subgroup with statistical significance ($P \leq 0.05$) when compared with the rest of subgroups (Fig. 6B).

4. Discussion

The present study indicated that there are some correlations between the shock wave intensity and the biological effect on cells *in vitro*. When cells were stimulated by ESWT with low energy (0.04–0.13 mJ/mm^2), shock wave could improve the expression of some angiogenic factors, such as *eNOS*, *Ang-1*, and *Ang-2*, and decrease the expression of apoptotic cytokines, which may lead to less apoptosis. When cells were stimulated by ESWT with high energy (0.16 mJ/mm^2), ESWT could reduce the expression of angiogenic factors, and increase the expression of apoptotic factors, which may induce cell apoptosis. Given the same shock wave energy, the effect of different numbers of shock wave impulses showed similar trend. Fewer impulses could increase the expression of cytokines and decrease the cell apoptosis, whereas more impulses could inhibit the expression of cytokines and induce cell apoptosis. ESWT could be deemed as a double-edged sword.

In the present work, the shock wave treatment with energy flux density ranging from 0.10–0.13 mJ/mm^2 and shock

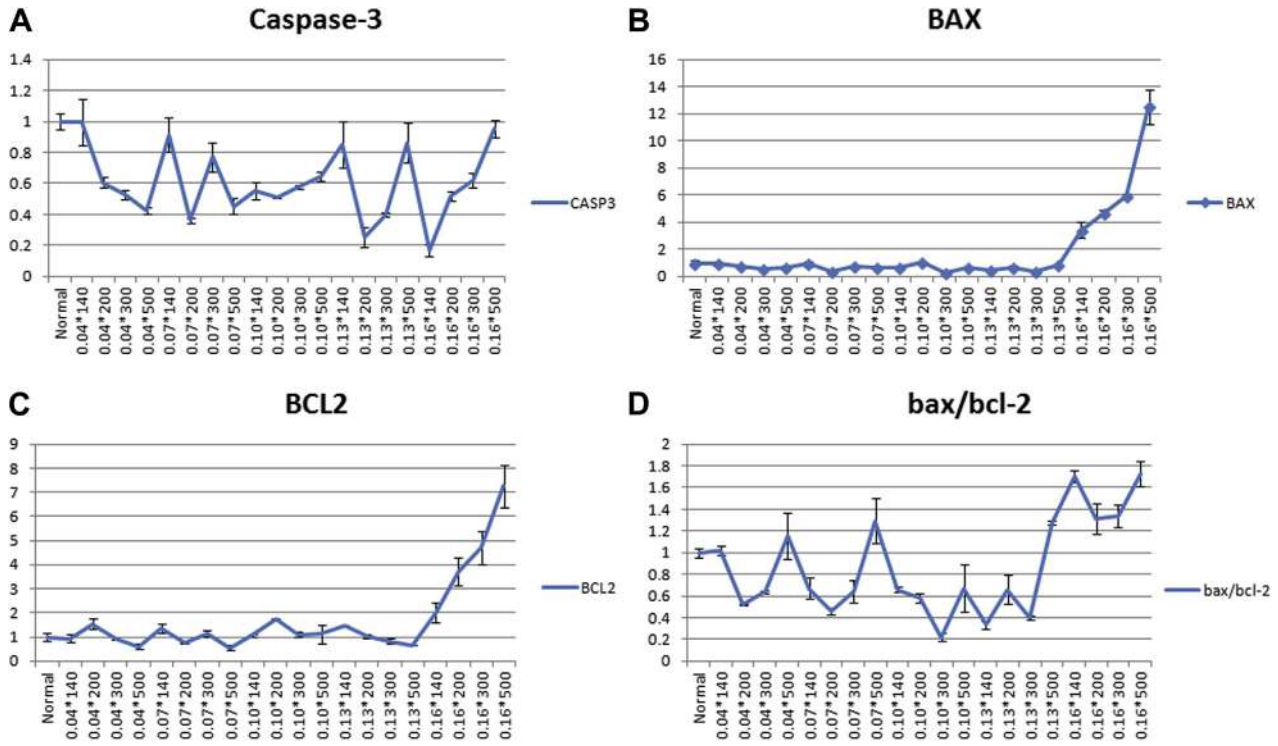

Fig. 5 – Detection of the apoptosis indicators by real-time PCR. Effect of different parameters on (A) caspase 3, (B) BAX, and (C) BCL-2. (D) The ratio of Bax and Bcl-2, the value was at low level in the area from 10 mJ with 300 impulses to 13 mJ with 300 impulses. (Color version of figure is available online.)

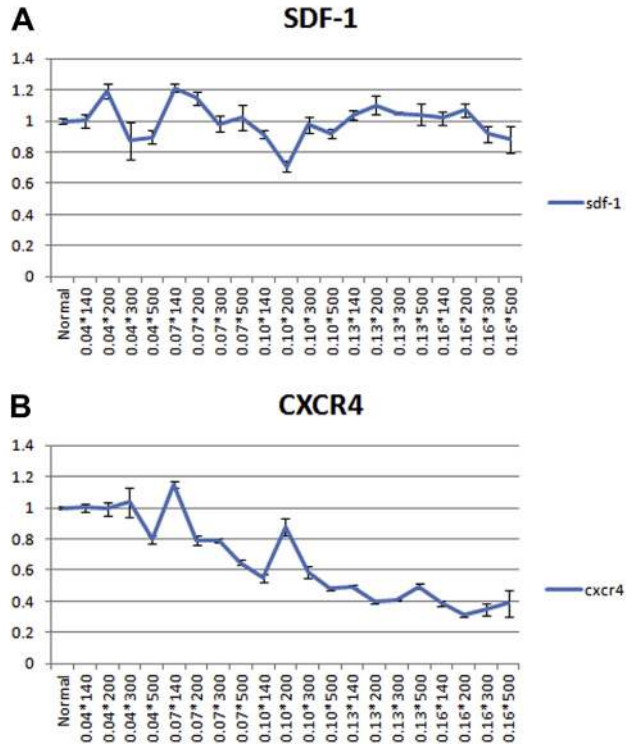

Fig. 6 – Detection of the expression of chemotactic factors by real-time quantitative PCR. Effect of different parameters on (A) SDF-1 and (B) CXCR4. (Color version of figure is available online.)

number ranging from 200–300 impulses showed more active proliferation activity, relatively higher and more effective expression of cytokines, relatively suppressing inflammation mediators and apoptosis, which was considered as the ideal and most suitable parameter for shock wave therapy to treat cells *in vitro*.

According to the reports by literature, each study used different parameters. ESWT with 500 [19–22], 300 [23], 229 [24], 200 [4,25,26], 181 [24],140 [24] impulses were reported, and energy flux density ranged from 0.05–0.15 mJ/mm^2. People have not got consensus on the parameter of shock wave to exert the strongest biological activity [27]. Although the parameter used varied, many studies applied shock wave with the intensity ranging from 0.09–0.11 mJ/mm^2 [3–6,19,21–23,25,28–31]. This fact is in accordance with our result.

Certainly, the effects of ESWT on different cytokines were not the same. Many cytokines are involved in the progress of reconstruction of blood supply. eNOS [32], VEGF [33], FGF [34], angiopoietin (Ang), and so forth, are well-known molecules with strong angiogenic effect. Our previous study suggested that shock wave therapy could increase blood perfusion in tissue by vasodilatation induced by enhanced activity of eNOS [6]. In the present study, we showed that *eNOS* expression of the cells increased significantly when EPCs were treated with shock wave therapy with proper energy. However, in the early stage after shock wave therapy (48 h), the expression of *VEGFa* and *FGF-2* decreased, suggesting that shock wave still may inhibit the expression of some cytokines to some extent.

Our study showed that messenger RNA expression of the two representative inflammation mediators decreased under the treatment with low-energy ESWT. FGF-2 is an inflammatory factor [35] in addition to an angiogenic factors, especially involved in chronic inflammation and responsible for fibrosis. But the meaning for the increased *FGF-2* by the action of shock wave with high energy flux density needs furthermore study. The suppression to inflammation mediators by shock wave indicated that the anti-inflammation is another mechanism of ESWT [36].

Other cytokines that increased with *eNOS* were *Ang-1* and *Ang-2*. The two cytokines are also regulators of angiogenesis. The specific biological effect of Ang-1 is chemotactic for endothelial cells [37]. The increase of *Ang-1* at early stage after shock wave treatment may contribute to the recruitment and aggregation of endothelial cells to the local site to form blood vessels. Ang-2 acts as a negative regulator of Ang-1/Tie2 signaling during angiogenesis, thereby controlling the responsiveness of endothelial cells to exogenous cytokines [38]. Interestingly, with the increase of shock wave energy level, the expression of *Ang-1* did not change a lot, but *Ang-2* reduced significantly, which lead to the increase of the *Ang-1/Ang-2* ratio. *Ang-1* and *Ang-2* levels showed conflicting results when cells were treated with ESWT with different parameters. This result implied that the effective expression of *Ang-1* increased when cells were treated with shock wave. Studies demonstrated that the decrease of the ratio *Ang-1/Ang-2* was related to progression of some diseases [39,40].

In the present study, we observed that the expression of *CXCR4* decreased significantly with the increase of shock wave energy intensity. This was not in accordance with some former researches. Aicher *et al.* concluded that shock wave therapy could increase the expression of *SDF-1*, the ligand of CXCR4 [41], and Yeh *et al.* suggested that shock wave therapy could increase the expression of *CXCR4* and *SDF-1* both in tissue samples. SDF-1 is the ligand of CXCR4 and plays an important role in the migration of hematopoietic stem cell to bone marrow [42]. However, the targets of shock wave therapy in the two studies were tissues, but it was EPCs in our study. So downward regulation of the chemotactic factors in stem cells should be furthermore verified in tissue and stromal cells.

Our study also indicated that shock wave could reduce the apoptosis when cells were treated with low-energy shock wave and induce apoptosis when cells were treated with high-energy shock wave. Researchers reported the increased apoptotic cells induced by high dose of shock wave application [43]. The energy density in the study was 0.08 mJ/mm^2, however, the number of shocks applied in the study was 500 and 1000, much more than the parameters we obtained based on the results of our study. Furthermore, in the cell proliferation assay, we also observed that cell proliferation was more active with the low energy intensity, whereas cell proliferation was inhibited obviously at the high energy intensity.

Besides number of shocks and energy flux density, frequency is another parameter of shock wave. There is little research done on the relation between shock wave frequency and bioactivity of cells. So the relationship between the effect and frequency of shock wave is still unclear. In our studies, we adopted the frequency of 2 shocks per second to avoid injuring cells by shock wave with high frequency.

To our knowledge, the present study is the first to transversely and systematically compare the cytobiological effects of shock wave with different energy intensities and impulses on cell proliferation, expression of cytokines, and apoptosis, and has obtained some interesting results, which may provide some useful clues for future study.

Last of all, our research was based on *in vitro* experiment. However, the situation would be more complicated *in vivo* because of the shielding effect of living tissue and characters of different tissues. The actual effects of ESWT should be confirmed by furthermore studies *in vivo*.

5. Conclusions

The present study used the real-time PCR and MTT assay as screening tools to study the effect of the shock wave with different parameters on the bioactivity of EPCs. According to the findings of our study, we believed that 0.10–0.13 mJ/mm^2 with 200–300 impulses was the most suitable parameter for shock wave therapy to treat EPCs, and may exhibit the strongest bioactivity in EPCs. We also preliminarily discussed the mechanism of shock wave therapy in improving the survival of ischemia tissue and provided some clues for future researches.

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The authors declare no other competing financial interests.

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