Effect of Extracorporeal Shock Wave Therapy Parameters on the Production of Angiogenesis Factors in Human Umbilical Vein Endothelial Cells

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Abstract
Extracorporeal shock wave therapy (ESWT) can promote the angiogenesis in the ischemic tissue. We hypothesized that the effect of ESWT on angiogenesis depended on the energy density, shot frequency, total irradiation energy and irradiation direction on the target cells. Thus, this study would analyze the physical mechanism of ESWT in promoting the expression of VEGF and eNOS from the aspects of cavitation effect, irradiation area, and direct flow. Human umbilical vein endothelial cells (HUVECs) were cultured and divided into 4 groups according to different ESWT energy density. Subsequently, each group was further divided into 4 subgroups based on the different shot frequencies, resulting in a total of 16 subgroups. ESWT vertical irradiation was adopted in cells of these 16 subgroups. In addition, the mRNA expression of vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS) was detected through Reverse Transcription-Polymerase Chain Reaction (RT-PCR), so as to obtain the optimal ESWT energy density and shot frequency on HUVECs. Afterwards, the influence of the total ESWT energy value on the eNOS mRNA expression quantity was calculated based on the ESWT energy density and shot frequency. After obtaining the optimal ESWT energy density and shot frequency through the above-mentioned methods, the ESWT was adjusted to irradiate cells in the slanted direction; besides, the production of VEGF and eNOS was compared with that under vertical irradiation, thus obtaining the effect of ESWT irradiation direction on the mRNA expression of VEGF and eNOS.

Key words: Extracorporeal Shock Wave Therapy, Angiogenesis Factors, Human Umbilical Vein Endothelial Cells.

Efecto de los Parámetros de la Terapia de Ondas de Choque Extracorpóreas Sobre la Producción de Factores de Angiogénesis en Células Endoteliales de Vena Umbilical Humana

Resumen
La terapia de ondas de choque extracorpóreas (ESWT) puede promover la angiogénesis en el tejido isquémico. Nuestra hipótesis fue que el efecto de la ESWT en la angiogénesis dependía de la densidad de energía, la frecuencia de disparo, la energía de irradiación total y la dirección de irradiación en las células objetivo. Por lo tanto, este estudio analizaría el mecanismo físico de ESWT para promover la expresión de VEGF y eNOS a partir de los aspectos del efecto de cavitación, área de irradiación y flujo directo. Las células endoteliales de la vena umbilical humana (HUVEC) se cultivaron y se dividieron en 4 grupos según la densidad de energía de ESWT diferente. Posteriormente, cada grupo se dividió en 4 subgrupos según las diferentes frecuencias de disparo, lo que dio como resultado un total de 16 subgrupos. La irradiación vertical ESWT se adoptó en las células de estos 16 subgrupos. Además, la expresión del ARNm del factor de crecimiento endotelial vascular (VEGF) y del óxido nítrico síntesis endotelial (eNOS) se detectó a través de la reacción en cadena de la polimerasa de transcripción inversa (RT-PCR), para obtener la densidad de energía ESWT óptima y la frecuencia de disparo HUVECs. Posteriormente, se calculó la influencia del valor de energía de ESWT total en la cantidad de expresión del ARNm de eNOS basándose en la densidad de energía de ESWT y la frecuencia de
disparo. Después de obtener la densidad de energía y la frecuencia de disparo de ESWT óptimas a través de los métodos mencionados anteriormente, el ESWT se ajustó para irradiar las células en la dirección inclinada; además, la producción de VEGF y eNOS se comparó con la irradiación vertical, obteniendo así el efecto de la dirección de irradiación de ESWT en la expresión de ARNm de VEGF y eNOS.

Palabras clave: Terapia de Ondas de Choque Extracorpóreas, Factores de Angiogénesis, Células Endoteliales de Vena Umbilical Humana.

1. Introduction

ESWT is a kind of vertical sound wave that can propagate in water or in soft tissue like ultrasound [1]. The tension generated by ESWT can produce cavitation effect, resulting with a large number of cavitation bubbles in the medium. With the effect of seismic waves, the cavitation bubbles oscillate non-linearly and expand gradually, making their radius expand. In the process of cavitation collapse, strong shock waves can be generated and high-speed microjet can be formed to act on tissues, cells and biomacromolecules, which can produce corresponding biological effects.

ESWT can be used in lithotripsy and in treating diseases such as nonunion, tendinitis and ischemic osteonecrosis [2-4]. Some research reported that, ESWT could induce angiogenesis in the swine myocardial ischemia model [5-6], and improve the myocardial perfusion and clinical symptoms in patients with severe coronary heart disease (CHD) [7-8], which can be utilized to treat ischemic diseases such as angina and ischemic heart failure [9-10]. ESWT has been recognized as a promising method to promote angiogenesis [11-12].

However, the precise mechanism of ESWT in promoting angiogenesis remains unclear at present. Under natural status, there are a large number of micro-sized vacuoles in human tissues, which can sharply swell to the millimeter scale under the action of ESWT. The micro-jet will be produced when these vacuoles collapse, which will act on the surrounding tissues and cells to produce the rapid and transient shear force [13]. The local stress that ESWT stimulates the endothelial cell membrane can enhance angiogenesis, which is similar to the fluid shear stress in blood vessel [14]. The parameters, like ESWT dose, can affect the effect of shear force on endothelial cells [15].

We hypothesized that, ESWT depended on the energy density, shot frequency, total irradiation energy, and irradiation direction on the target cells to exert its angiogenesis effect. Therefore, this research aimed to investigate the dose-effect relationships of the energy density, shot frequency, total irradiation energy, and irradiation direction of ESWT with the biological effect, analyze the physical mechanism of ESWT in promoting angiogenesis, and determine the optimal parameters of ESWT to treat the cells cultured in vitro.

2. Materials and methods

2.1. Cell culture

(1) Cells for experiment: human umbilical vein endothelial cells (HUVECs) (Wuhan Procell Life Technology Co., Ltd, China).

(2) Main experimental apparatus

Bechtop (Suzhou Antai Air Technology Co., Ltd, China), low-temperature high-speed centrifuge (3-18K, Sigma, Germany), inverted phase contrast microscope (BH1, Olympus, Japan), and CO2 incubator (17-AC, SANYO, Japan).

(3) Main reagents

Anti-eNOS antibody (Sigma, USA), anti-VEGF antibody (Sigma, USA), β-actin antibody (TransGen Biotechnology Co., Ltd, China), secondary antibody (rabbit anti-mouse IgG, Invitrogen, USA), thiazolyl blue (MTT, Sigma, USA), fetal bovine serum (FBS, Gibco, USA), 0.25% trypsin (Gibco, USA), DMEM (Gibco, USA), Matrigel (Vigorousbi, Beijing), and all the other reagents were at domestic analytical purity.

(4) Culture and grouping of HUVECs

HUVECs were cultured in the RPMI-1640 medium (containing 10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin) in an incubator under the conditions of 5% CO2 and 37°C. Subsequently, 0.25% trypsin was used for digestion and passage. HUVECs at exponential phase were inoculated at the 4-well plates at the density of 1×106/well, and placed into the 5% CO2 incubator. When cells had grown to 70%-80% of conflated, the medium was replaced with the 1% FBS-containing DMEM to culture for another 24 h, so as to achieve cell synchronization. Afterwards, cells were divided into 4 groups according to different energy densities, including 0.04 mJ/mm2, 0.09 mJ/mm2, 0.12 mJ/mm2 and 0.16 mJ/mm2 groups. Then, each group was further divided into 4 subgroups based on the ESWT shot frequency, namely, 160 shots, 250 shots, 350 shots and 500 shots, resulting in a total of 16 subgroups.
2.2. ESWT treatment on HUVECs

(1). Vertical ESWT irradiation on HUVECs
The Dornier AR2 ESWT (Dornier MedTech, Germany, energy density of 0.005-0.32 mJ/mm²) was used for ESWT treatment on the target cells. The ESWT could generate settled electromagnetic shock wave with about 1 cm of diameter and 4 cm of effective length. The sterile ESWT probe was vertically immersed into the well plate and positioned to just contact the HUVEC mediator surface. The distance between the ESWT probe and the HUVEC layer on the dish bottom was about 1.5 cm. HUVECs were exposed to ESWT and incubated in the condition of static status in the CO2 incubator at 37 °C. Control cells were prepared in the same manner except that for ESWT treatment. HUVECs were harvested in a time-dependent manner after ESWT exposure for RT-PCR.

(2). Slanted ESWT irradiation on HUVECs
The irradiation direction of ESWT acting on the target cells was adjusted. The ESWT probe was adjusted to irradiate the target cells at an angle of about 45 degrees. The optimal ESWT energy density and shot frequency obtained from the first step of vertical irradiation were used to treat the HUVECs.

![Diagram of ESWT on target cells at the vertical and slanted irradiation directions. A) Vertical ESWT irradiation on target cells; B) slanted ESWT irradiation on target cells.](#)

2.3. mRNA expression levels of eNOS and VEGF in HUVECs of each group detected by RT-PCR
The influence of ESWT on the mRNA expression of vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS) was detected through RT-PCR. RNA was extracted from 2 groups of HUVECs, purified, and synthesized into cDNA in accordance with the reverse transcription kit instruction (MBI). Later, the primers were designed using the primer premier 5.0 software and synthesized by Sangon, as shown below: VEGF (sense strand): 5’-TCACAGGTACAGGGATGAGGACAC-3’ and (anti-sense strand): 5’-CAAAGCACAGCAATGTCCTGAAG-3’;
eNOS: (sense strand): 5’-AAAGACAAGGCCAGCAGTGGAAAT-3’ and (anti-sense strand) 5’-TCCACGATGGTCATTGCTGGC-3’;
GAPDH (sense strand): 5’-GCACCGTCAAGGGGAC-3’ and (anti-sense strand): 5’-TGTTGAAGACGACGTCAG-3’. The reaction conditions were shown below: at 94°C for 2 min, at 94°C for 30 s for 35 loops, at 58°C for 30 s, at 72°C for 1 min, and at 72 °C for 8 min. The length of the amplified segment was 496 bp, and PCR reaction was performed under the set conditions, followed by
agarose gel electrophoresis (AGE), gel imaging, scanning, result observation under the ultraviolet lamp, photo taking and recording. The GAPDH gene was used as the internal reference gene, and the relative expression (RQ) of the target gene in VEGF sample was calculated by the 2-ΔΔCt method in accordance with literature [16], as shown below:

\[ RQ = 2^{-\Delta\Delta Ct} \] (1)

Where ΔΔCt = target gene ΔCt in the test sample - target gene ΔCt in control group, while ΔCt = target gene Ct - internal reference gene Ct.

2.4. Statistical analysis

All experimental data were expressed as mean ± standard deviation, and the SPSS21.0 statistic software was adopted for statistical analyses. The means of multi-group samples would be compared through analysis of variance, while inter-group pairwise comparison was conducted using SLD-q test, and the Pearson correlation analysis was used for correlation analysis. A difference of P < 0.05 was deemed as statistically significant.

3. Results

3.1. Effects of ESWT energy density and shot frequency on VEGF mRNA expression quantity

Figure 3 shows the influence of ESWT energy density and shot frequency on the VEGF mRNA expression quantity. Under low ESWT energy density (0.04-0.12 mJ/mm²), the VEGF mRNA expression quantities in most subgroups were higher than that in control group, and the maximum expression quantity could be attained at the energy density of 0.09 mJ/mm² and shot frequency of 250 shots (P < 0.05); while the expression quantities in 350 shots and 0.09 mJ/mm² group, and 250 shots and 0.12 mJ/mm² group had accounted for the second and third places, respectively. When the energy density reached 0.16 mJ/mm², the VEGF mRNA expression quantity was almost not increased.

3.2. Effects of ESWT energy density and shot frequency on eNOS mRNA expression

ESWT energy density and shot frequency would affect the eNOS mRNA expression quantity. Under the same shot frequency, the energy density had displayed a rule similar to normal distribution; typically, the energy density of 0.09 mJ/mm² could evoke the greatest eNOS mRNA expression quantity at the shot frequencies of 350 and 500 shots; while the energy density of 0.12 mJ/mm² could evoke the greatest eNOS mRNA expression quantity at the shot frequencies of 160 and 250 shots. At the energy density of 0.16 mJ/mm², the eNOS mRNA expression quantity was almost not increased under any shot frequency. Under the same energy density, the shot
frequencies of 250 and 350 shots could greatly promote the eNOS mRNA expression quantities. Our experiment suggested that, a low or high ESWT shot frequency was not good for the eNOS mRNA expression. Under the conditions of this experiment, the optimal combination of energy density and shot frequency was 0.09 mJ/mm² and 350 shots (P < 0.05).

\[ J = j \times n \]  

(2)

Where \( J \) is the total ESWT energy in each subgroup, \( j \) is the energy density in each shot of ESWT, and \( n \) is the shot frequency in each group.

Took eNOS as an example, the relationship between the total ESWT energy and the eNOS mRNA expression quantity is displayed in Fig.4. From the overall trend, the eNOS mRNA expression quantity was increased with the increase in total ESWT energy, which peaked at the total energy of 31.5 mJ/mm² and was decreased thereafter.

Figure 3 Effects of ESWT energy density and shot frequency on eNOS mRNA expression

As could be observed from the data, even the same ESWT energy density and shot frequency would exert different promotion effect on different factors. For instance, ESWT at 0.09 mJ/mm² and 250 shots had the highest effect on promoting VEGF mRNA expression among all subgroups, but such combination ranked the 4th place among all subgroups regarding its effect on promoting the eNOS mRNA expression.

It could also be discovered from the experiment that, under our experimental conditions, in addition to the ESWT energy density \( j \) and shot frequency \( n \), the total ESWT energy \( J \) also had certain influence on promoting the mRNA expression quantities of different factors.
3.3 Effects of ESWT irradiation directions on the mRNA expression quantities of VEGF and eNOS

To investigate the influence of ESWT irradiation direction on promoting the expression of VEGF and eNOS, we had adjusted ESWT to irradiate HUVECs at the slanted direction. For VEGF, the ESWT parameters of 0.09 mJ/mm² and 250 shots were adopted; while for eNOS, the ESWT parameters of 0.09 mJ/mm² and 350 shots were utilized. Afterwards, the mRNA expression of VEGF and eNOS were detected through the RT-PCR, and were compared with those under vertical irradiation.

![Graph A](image1.png)

**Different radiation directions**

- VEGF mRNA expression under slanted ESWT irradiation was greater than that under vertical irradiation (Fig. 5A).
- eNOS mRNA expression under slanted ESWT irradiation was greater than that under vertical irradiation (Fig. 5B).

**Figure 5.** Effects of ESWT irradiation directions on the mRNA expression quantities of VEGF and eNOS

The ESWT irradiation direction would affect the mRNA expression of VEGF and eNOS. Specifically, the VEGF mRNA expression under slanted ESWT irradiation was greater than that under vertical irradiation (Fig. 5A), while the eNOS mRNA expression under slanted ESWT irradiation was greater than that under vertical irradiation (Fig. 5B).

4. Discussion

This study indicated that, ESWT energy density was correlated with the expression of angiogenesis factors VEGF and eNOS. ESWT irradiation at a low energy density (0.04-0.12 mJ/mm²) on cells could increase the expression of angiogenesis factors such as VEGF and eNOS; however, ESWT irradiation at a high energy density (0.16 mJ/mm²) had no obvious effect on promoting the expression of angiogenesis factors, instead, it may even reduce the expression of related factors. High energy density (0.16 mJ/mm²) ESWT irradiation target cells may affect cell viability. Chang Hoon Ha, et al. used the ESWT of 0.16 mJ/mm² and 1000 shots to irradiate vascular endothelial cells, leading to a large number of cell death [13]. Therefore, we may have caused cell death when we irradiated HUVECs with high-energy ESWT, resulting in decreased expression of angiogenic factors.
When the ESWT energy density was the same, the ESWT shot frequency would also affect the expression of VEGF and eNOS, and a low or high shot frequency was not good for the expression of VEGF and eNOS. Xiongliang Zhang et al. used ESWT with different shots frequency on endothelial progenitor cells and found that, more shots could inhibit the expression of cytokines and induce cell apoptosis [15]. Hon-Kan Yip, using ESWT with the energy density of 0.09 mJ/mm² to bone marrow-derived mononuclear cells (BMDMNCs) to enhances the formation of vascular endothelial growth factor (VEGF) [17], the shots frequency was 140 shots, 280 shots and 560 shots, and the results showed that the VEGF expression under 280 shots was higher than 140 shots and 560 shots, which was similar to our results.

Our findings suggested that, even the same ESWT energy density and shot frequency would had different effects on promoting the production of different angiogenesis factors. For VEGF factor, the optimal combination of energy density and shot frequency was 0.09 mJ/mm² and 250 shots; while those were 0.09 mJ/mm² and 350 shots for eNOS. Nonetheless, no consensus has been reached regarding what kinds of ESWT energy density and shot frequency can better promote the production of VEGF and eNOS in HUVECs. Yet the ESWT energy density used in numerous studies ranges from 0.09-0.11 mJ/mm² [18-19], which is consistent with our results.

Apart from the ESWT energy density and shot frequency, the total energy is another parameter of ESWT. Little research is available on the relationship between total ESWT energy and cellular bioactivity. This study had analyzed the influence of total ESWT energy, and discovered that the expression of eNOS mRNA increased accordingly with the increase of the total ESWT energy; typically, the eNOS mRNA expression peaked at the total energy of 31.5 mJ/mm², which decreased thereafter.

In addition, the present experiment also discovered that, the ESWT irradiation direction would also affect the expression of VEGF and eNOS. Under slanted ESWT irradiation, the expression of VEGF and eNOS was slightly improved compared with that under vertical direction, which might be related to the different physical effect produced under different ESWT irradiation directions, such as cavitation effects, irradiation areas and direct flows, thus affecting the expression of related factors.

1) Cavitation effect. Vertical and slanted ESWT irradiation would generate the cavitation bubbles on the cells. Under the action of ESWT, the micro-sized vacuoles would swell to the millimeter scale, and micro-jet would be produced when these vacuoles collapsed, which would act on the surrounding tissues and cells to produce the rapid and transient shear force [20]. This might account for one of the physical causes of ESWT in promoting the enhanced expression of angiogenesis factors in cells. Typically, the micro-jet directions were different under different irradiation directions, which would also generate different shear force directions on the cell surface, and this might be the reason why slanted irradiation could enhance the expression of various factors.

![Diagram](Figure 6 Micro-jet will be produced when the vacuoles collapse, which will generate the shear force on the surrounding cells)

(2) Irradiation area. Compared with vertical irradiation, the area under slanted irradiation is greater. At the time of vertical ESWT irradiation, the area of the irradiated cells can be calculated according to:

\[
S_V = \pi \left( \frac{R}{2} \right)^2 \quad (3)
\]

Where \(SV\) is the area under vertical irradiation, and \(R\) is the radius of vertically irradiated area.

The irradiated cell area can be calculated as follow at the time of slanted ESWT irradiation:

\[
S_S = \sqrt{2\pi} \left( \frac{R}{2} \right)^2 \quad (4)
\]
As a result, more cells can be irradiated under slanted irradiation, thus promoting the expression of vascular factors.

(4) Direct flow. Direct flow is referred to as the phenomenon in which sound wave flows in liquid along the sound propagation direction. At the sound wave energy density of 0.5 W/cm², the flow of direct flow vertical to the plane of vibration can be seen under the naked eye, and the flow rate is about 10 cm/s. The energy density in ESWT is low, but it can also form certain liquid flow on the cell surface, which will produce the shear force on the cell surface, thus contributing to the expression of related vascular factors.

Figure 7 Direct flow will produce the shear force on the cell surface

5. Conclusions

(1) ESWT energy density and shot frequency are correlated with the bioactivity of human umbilical vein endothelial cells (HUVECs). With regard to promoting the synthesis of VEGF, the optimal combination of energy density and shot frequency is 0.09 mJ/mm² and 250 shots, whereas that is 0.09 mJ/mm² and 350 shots for promoting eNOS synthesis.

(2) The total ESWT energy also affects the production of angiogenesis factors. The eNOS mRNA expression quantity is increased with the increase in the total ESWT energy in each subgroup, which peaks at the total energy of 31.5 mJ/mm² and is decreased thereafter.

(3) The ESWT irradiation direction can also affect the expression of VEGF and eNOS. Typically, the expression of VEGF and eNOS under slanted ESWT irradiation is increased compared with that under vertical direction.

References


