Experimental Study

Effect of Shock Wave on Vascular Lesions in Diabetic Rats

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Background: Diabetes is one of the most common diseases in today's society. Diabetes can cause multiple vascular lesions in the body, renal insufficiency, blindness, and so on. However, the evidence concerning the role of extracorporeal shock wave therapy in diabetic vascular disease is insufficient.

Objectives: Observation of the effect of shock wave on vascular lesions in diabetic rats.

Study Design: This study used an experimental design.

Setting: The research took place in the laboratory research center at The Third Military Medical University.

Methods: Eighteen healthy adult male Sprague Dawley rats were randomly divided into 3 groups: normal control group (group A), diabetic group (group B), and diabetes + shock wave treatment group (group C). Groups B and C were established by intraperitoneal injection of streptozotocin 60 mg/kg to demonstrate a diabetic rat model. Shock wave treatment was performed on the left lower extremity femoral artery in group C for 1 week (T1), 2 weeks (T2), 3 weeks (T3), and 4 weeks (T4) while the other 2 groups were reared normally. At the end of T4 shock wave treatment, the femoral arteries of each group were observed under an electron microscope. The expression of vascular endothelial growth factors (VEGF), endothelial nitric oxide synthase (eNOS), and angiotensin type 1 (AT1) were measured by western blot, and the changes of VEGF expression were detected by real-time polymerase chain reaction.

Results: The VEGF and eNOS in group C were higher than those in group B ($P < 0.05$). The AT1 of the rats in the B and C groups was significantly higher than that in the A group ($P < 0.05$), but the C group was significantly lower than the B group ($P < 0.05$). After shock wave therapy, the surface of vascular endothelium in group C was flatter and smoother than that in group B, and the endothelial basement membrane and foot process were relatively tight.

Limitations: Potential mechanisms that underlie the relationship between vascular dysfunction and diabetic neuropathy pain were not examined in this study.

Conclusions: Shock wave may promote the formation of new blood vessels and improve vasomotor function by upregulating VEGF, eNOS, and downregulation of AT1 in diabetic rats and improve the damage of blood glucose to blood vessels to some extent.

Key words: Shock wave, diabetic rats, vascular dysfunction, neovascularization

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There are more than 400 million people with diabetes worldwide, and diabetes is one of the most common diseases in today's society (1). Diabetes can cause multiple vascular lesions in the body, and cause serious consequences such as amputation, renal insufficiency, and blindness (2-5). These complications not only seriously affect the health of the patient, but also add a heavy social burden.

Although medical treatment methods have gradually improved and bioengineering has developed rapidly, there is still no effective treatment for diabetic vascular disease. Therefore, the exploration of new treatments for diabetic vascular disease is crucial. The mechanism of diabetes lesions has not been thoroughly studied so far, and the mechanisms proposed include: hyperglycemia-dependent mechanisms, such as the formation of glycosylation end products; a vasoactive hormone system that regulates blood pressure, such as the renin-angiotensin-aldosterone system (RAAS) and the endothelium system; and a nitrogen oxide synthase signaling pathway (6-9). Therefore, the current treatment methods are mainly to control blood sugar and blood pressure reasonably, but it can only delay the progress of complications instead of improving the existing vascular lesions. In recent years, in addition to the application of nonurinary calculi, extracorporeal shock wave therapy (ESWT) can produce acoustics through its mechanical transmission, affecting biological reactions in vivo (10), such as promoting the release of endogenous angiogenic factor (vascular endothelial growth factors [VEGF]) in vascular endothelial cells and promoting neovascularization (11). According to the study, blood flow perfusion is restored after shock wave action, and the effects of microcirculatory hypoxia and related ischemic organ dysfunction are improved (12). However, it is unclear whether ESWT can improve diabetic vascular disease. Therefore, this study intends to establish a diabetic rat model to observe the effect of ESWT on the lower extremity femoral artery and explore its possible mechanism.

Methods

Experimental Animals and Grouping

Eighteen healthy adult male Sprague Dawley rats, weighing 250 to 300 g, were purchased from the Animal Laboratory of the Third Military Medical University (animal use license number: SCXK (R) 2012-0005). Rat were kept in cages and were free to eat and drink. Animals were divided into normal control group (group A, n = 6), diabetes group (group B, n = 6), and diabetes + shock wave treatment group (group C, n = 6) by random number table method. All procedures in this study were in accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines and approved by the Institutional Animal Care and Use Committee of the Third Military Medical University.

Model Establishment

Diabetes Model

Rats in groups B and C were intraperitoneally injected with streptozotocin 60 mg/kg (STZ, Sigma, St. Louis, MO), and rats in group A were intraperitoneally injected with the same dose of normal saline solution. The blood glucose concentration of the tail vein blood samples was measured in the 3 groups of rats before fasting (after 12 hours of fasting) and 7 days after injection (fasting for 12 hours). After 7 days of injection in groups B and C, the blood glucose concentration in the tail vein was >16.7 mmol/L, which was regarded as diabetic rats. The blood glucose concentration in the tail vein of both groups B and C exceeded the standard of diabetic rat model, and 18 rats were subjected to the next experiment.

Shockwave Treatment

We set the time before modeling to T0. Shock wave treatment of the left lower extremity femoral artery in group C was performed 1 week (T1), 2 weeks (T2), 3 weeks (T3), and 4 weeks (T4) after successful modeling. Shock wave treatment parameters are frequency 10 to 15 Hz, energy 1.0 to 3.0 bar. Before treatment, the distribution of the left femoral artery of the rat was treated with skin preparation, and the iodophor was routinely disinfected. The distribution of the left femoral artery was used as the treatment area, and 6,000 strokes were given for each treatment. After the last treatment, the left lower extremity femoral artery was taken for subsequent testing. All rats in group A survived. Of the rats in group B, one died in T2, and rats in group C, one died in T4.

Electron Microscopic Observation of Blood Vessels

We placed the left lower extremity femoral artery in glutaraldehyde (3%) and osmium tetroxide (1%). After dehydration by gradient alcohol, epoxy resin 812 (Electron Microscopy Sciences, USA) was soaked and embedded to make ultrathin sections of tissue. After counterstaining with uranyl acetate (30 minutes) and lead citrate (10 minutes), a 15,000-fold observation was observed by fluoroscopy.

RNA Extraction and Real Time-Polymerase Chain Reaction Detection of VEGF

The total RNA of the femoral artery was extracted by the Trizol (Thermo Fisher Scientific, USA) method and reverse transcribed into cDNA. Primer design VEGF

was sense 5'CGAGGCAGCTTGAGTTAAAC 3' antisense 5'TCCGGTGAGAGGTCTAGTTC 3'. We followed the steps of Maxima SYBR Green/ROX qPCR Master Mix (2×) (Thermo Fisher Scientific, USA), 3 wells per concentration. The polymerase chain reaction was predenatured at 96°C for 6 minutes, denatured at 96°C for 30 seconds, annealed at 57°C for 30 seconds, extended at 72°C for 30 seconds, 40 cycles, and finally extended at 72°C for 10 minutes.Calculate the $2^{-\Delta\Delta CT}$ value.

Western Blot (WB) Detection of Femoral Artery VEGF, eNOS, and AT1

The volume ratio of radioimmunoprecipitation (RIPA) lysate to Phenylmethylsulfonyl fluoride (PMSF) was mixed at 100:1, the tissue was fully lysed, placed in a centrifuge at 1°C for 15 minutes at 4°C, and the supernatant was removed. According to the BAC protein concentration determination kit (Thermo Fisher Scientific, USA), the sample protein concentration was measured. After vertical electrophoresis, the membrane was transferred for 1.5 hours, blocked for 2 hours, and the primary antibody was treated at 4°C, overnight (VEGF: RD Biosciences, San Diego, CA, US, diluted 1:1000 endothelial nitric oxide synthase [eNOS]: Novus Biologicals, USA, diluted) 1:1000; angiotensin type 1 [AT1]: Santa Cruz Biotechnology, USA, diluted 1.2: 1000). The secondary antibody was incubated for 1 hour at room temperature (diluted 1:1000), the ECL luminescence solution was chemiluminescence, and the film was exposed.

Statistical Analysis

Statistical analysis was performed using the SPSS 20.0 statistical package (IBM Corporation, Armonk, NY). Measurement data were shown by mean ± standard deviation. One-way analysis of variance was used for comparison between groups, and Least-Significant Difference method was used for pairwise comparison. $P < 0.05$ was considered statistically significant.

Results

Assessing the Expression of Rat Femoral Artery Vegf, Enos, And At1 By Western Blot

Diabetes can increase the AT1 of the femoral artery. The blood vessels of the B and C groups were significantly increased in the AT1 and A groups ($P < 0.05$), but the AT1 was significantly decreased after the shock wave treatment. The AT1 in the C group was significantly lower than that in the B group ($P < 0.05$). Diabetes caused a decrease in eNOS and VEGF in the femoral artery. The levels of eNOS, VEGF in the B and C groups were significantly lower than A group ($P < 0.05$), but group C was higher than group B ($P < 0.05$) (Fig. 1).

Real-Time Polymerase Chain Reaction Detection of VEGF Expression in Rats

Shock wave treatment can significantly improve the VEGF of femoral artery in diabetic rats. Compared

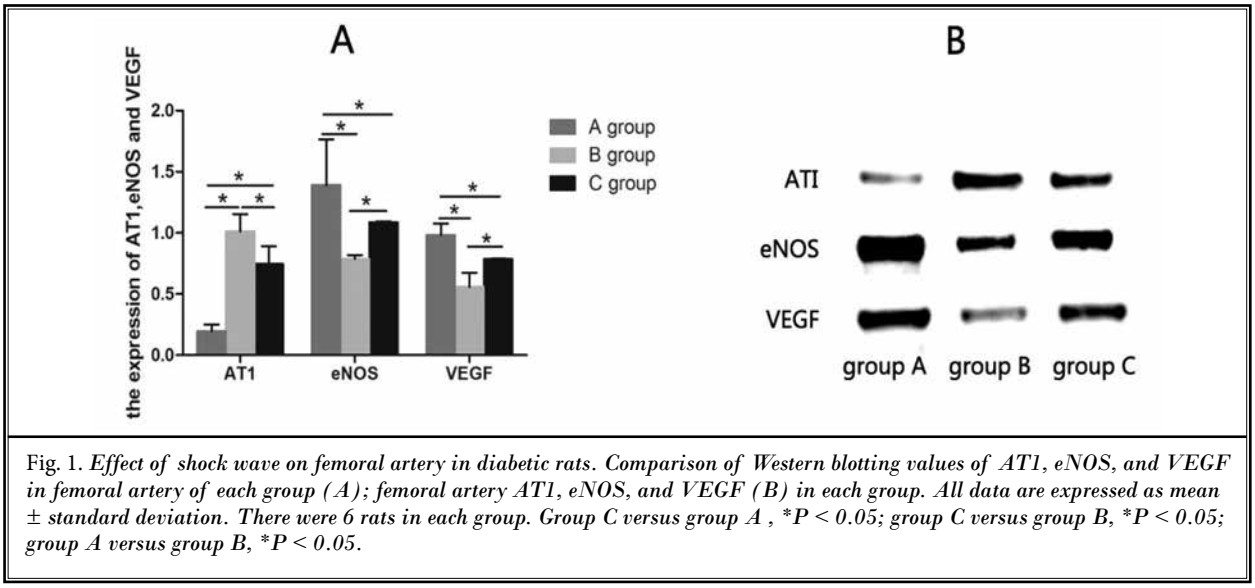

Fig. 1. *Effect of shock wave on femoral artery in diabetic rats. Comparison of Western blotting values of AT1, eNOS, and VEGF in femoral artery of each group (A); femoral artery AT1, eNOS, and VEGF (B) in each group. All data are expressed as mean ± standard deviation. There were 6 rats in each group. Group C versus group A , *P < 0.05; group C versus group B, *P < 0.05; group A versus group B, *P < 0.05.*

Table 1. *Comparison of VEGF expression in femoral artery of each group ($\bar{X} \pm S$)*

Group	Group A	Group B	Group C	F value	P value
VEGF	1.044 ± 0.038	0.177 ± 0.007*†	0.456 ± 0.035*	1290.783	0.000

The VEGF in femoral artery of group A was obviously higher than that in group B and C. Group C was higher than group B. Six rats per group. Group A versus group B and C, *P < 0.05; group B versus group C, †P < 0.05.

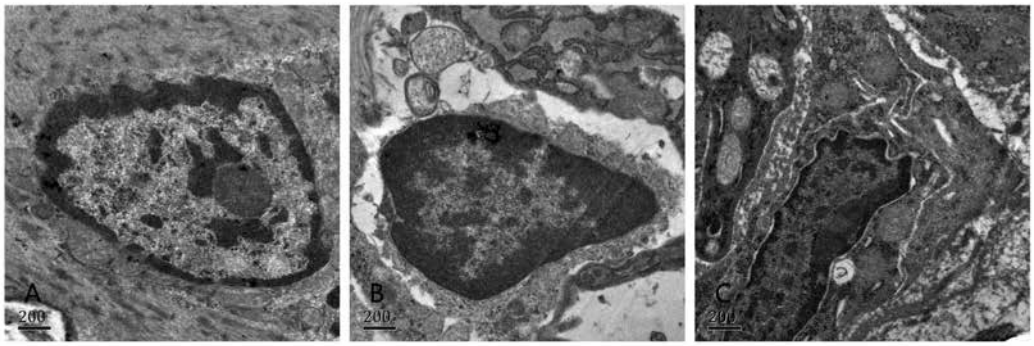

Fig. 2. *Effect of show wave on the ultrastructure of femoral artery in diabetic rats. Comparison of femoral artery ultrastructure in each group (15000 times). A-C: Ultrastructure of femoral artery in rats of group A, B, and C, respectively. Six in each group.*

with group A, VEGF was significantly decreased in group B and C ($P < 0.05$), but group C was higher than group B ($P < 0.05$) (Table 1).

Electron Microscopy Detection of Femoral Artery Ultrastructure

The morphology and structure of the femoral artery endothelial cells in group A were normal, no vacuolar edema, and the cells were closely connected (Fig. 2A). Most of the endothelial cell envelopes of group B were absent and endothelial cells were shed, the intercellular connections were loose or even broken, intracellular mitochondria were swollen and cytoplasmic vacuolization (Fig. 2B). The endothelial cells of group C were partially enveloped, and most of the tight junctions disappeared into gap junctions. The mitochondria showed slight swelling, sputum disorder, and vacuolization of cytoplasm. Endothelial cell damage was improved compared with group B (Fig. 2C).

Discussion

Vascular lesions are the main complication of patients with diabetes. Large blood vessels can involve coronary arteries, cerebrovascular vessels, and peripheral blood vessels. Microvascular lesions are common in kidney, retina, et cetera, which are the causes of myocardial infarction, stroke, gangrene or amputation, renal insufficiency, blindness, and others in patients with diabetes. Complications caused by these vascular diseases seriously affect the quality of life and health of patients, and even threaten the lives of patients. However, although today's medical methods are evolving with the rapid development of science and technology, there is no effective treatment other than to control blood sugar, delaying the progression of vascular disease.

A number of recent studies have shown that low energy shock waves can be applied to the treatment of musculoskeletal and cardiovascular diseases by exerting their biological effects (13-15). Animal experiments have shown that repeated effects of shock waves or short-term use can improve microcirculation (16,17). Treatment of ischemic organs and tissues with shock waves of appropriate energy can reduce inflammation and induce neovascularization (13,18). In the in vivo experiment of this study, the morphology and structure of the femoral artery endothelial cells in group A (control group) under electron microscope were normal, no vacuolar edema, and the cells were closely connected. In the diabetic rat model (group B), most of the femoral artery endothelial cell capsules were absent and endothelial cells were shed, the intercellular connections

were loose or even broken, intracellular mitochondria were swollen, there was sputum disorder and cytoplasmic vacuolization, corresponding to endothelial cell damage caused by diabetic vascular disease. After treatment with shock wave, the ultrastructure of blood vessels in group C were flatter and smoother than that in group B, and the connection between endothelial basement membrane and foot process was relatively close. It can be seen that shock wave therapy has a certain effect on the repair of vascular endothelial injury. The study by Oi et al (14) suggests that shock wave therapy can attenuate the carotid inflammatory response in the mouse carotid injury model and promote neointimal formation and smooth muscle cell proliferation.

In addition, some scholars found in vitro that ECSW can upregulate the expression of VEGF in human umbilical vein endothelial cells and rat bone marrow cells, and also promote the differentiation of bone marrow cells into endothelial cells (13,15). VEGF is a major factor in the synthesis of endothelial cells, and increases intracellular Ca2+ levels after binding to receptors and then promotes the proliferation and migration of endothelial cells through the phospho-C enzyme pathway, which mediates actin recombination and participates in neovascularization under physiological or pathological conditions. In this study, group B was downregulated compared with group A VEFG, suggesting that VEGF levels were downregulated in the high glucose state. Similarly, glomerular microarray results in patients with diabetic nephropathy showed a downregulation of VEGF mRNA levels (19). Combined with the results described earlier, it shows that the level of VEGF is lowered under the condition of hyperglycemia in diabetes. The VEGF level of group C rats after shock wave treatment was higher than that of group B. Shock wave could upregulate the expression of VEGF in femoral artery of diabetic rats. Combined with the results of electron microscopy, it is suggested that the promotion of vascular endothelial injury repair by shock wave therapy may be based on the regulation of VEGF expression level after shock wave treatment. In addition, it is worth noting that animal experiments suggest that VEGF levels are too high or too low to cause kidney disease (20,21). Therefore, the extent of the impact of shock waves on VEGF expression is also the focus of attention and our further research.

In the pathogenesis of diabetes, oxidative stress plays an indispensable role in diabetic angiopathy (3,5,22). Oxidative stress can cause eNOS to be decoupled, causing superoxide anion to damage the vascular endothelium and affecting the diastolic function of blood vessels. Under physiological conditions, eNOS is involved in the production of nitric oxide (NO), which in turn maintains the balance of endothelial cell redox signaling pathway. Therefore, downregulation of eNOS expression leads to downregulation of NO, which can cause endothelial cell damage (23,24). The results of group B electron microscopy and western blot confirmed this. The eNOS in group C was higher than that in group B. Combined with electron microscopy results, it was suggested that the shock wave treatment of diabetic rats increased the expression level of eNOS and promoted the repair of vascular endothelium.

AT1 receptors are widely distributed in the vascular system and rely mainly on binding to angiotensin 2. In diabetic mice with AT1 knockout, kidney inflammation and fibrosis are relatively mild (25). Drug intervention with the RAAS can protect against complications in diabetes (5). All of these studies have suggested that RAAS is closely associated with diabetic complications. In this study, the expression level of angiotensin receptor (AT1R) in diabetic rats (group B) was higher than that in group A, and the expression of AT1R in group C after shock wave treatment was downregulated. It is suggested that shock wave downregulates AT1R and antagonizes the role of RAAS in vascular lesions.

Conclusions

In summary, shock wave therapy may upregulate vascular VEGF and eNOS, downregulate AT1, and counteract the mechanism of endothelial damage in diabetic vasculopathy, which is beneficial to alleviate diabetic vascular disease and promote vascular remodeling. The clinical application of shock wave is noninvasive and has no side effects. At present, the main treatments are around the control of blood pressure and blood sugar, which gives them the possibility to use it in the treatment of diabetic angiopathy. However, this study is an animal experiment, and further clinical trials are still needed for practical verification.

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