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ANDROLOGY
Original Article

Effect of low-energy shockwave therapy on angiogenic factors in the penile tissue of diabetic rats

Düşük enerjili şok dalga tedavisinin diyabetik sıçanların penis dokusundaki anjiyogenez faktörlerine etkisi

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ABSTRACT

Objective: The aim of this study is to investigate the effect of low-energy shock wave therapy (LESWT) on angiogenesis factors at penile tissue in a diabetic rat model.

Material and methods: A total of 30 male Sprague-Dawley rats which were allocated into three equal groups were included study. Group 1 (control group) included 10 male rats which did not receive any treatment were randomly chosen to serve as normal control. The remaining rats were injected intraperitoneally with 60 mg/kg of streptozotocin (STZ) to induce diabetes. Diabetic rats were divided into two equal group which constituted diabetic control, and LESWT treatment (DM+LESWT) group. Each rat in the DM+LESWT group received L-ESWT therapy. Endothelial nitric oxide synthase (eNOS) and vascular endothelial growth factor (VEGF) mRNA expression levels of penile tissue were evaluated.

Results: Following STZ dosing eNOS level dropped in the diabetic control group relative to the control group. Statistically significant increase in eNOS levels were seen in the LESWT+DM group. Similarly, in the diabetic control group STZ treatment decreased VEGF levels, while in the LESWT+DM group VEGF nearly approached to baseline levels. However variations in VEGF levels were not statistically significant.

Conclusion: Mechanism action of ESWT in the penile tissue seems to involve angiogenic factors.

Keywords: Angiogenesis; animal study; penis; shockwave therapy.

ÖZ

Amaç: Düşük enerjili şok dalga tedavisinin (LESWT) diyabetik rat modelinde anjiyogenez faktörlerine etkisini araştırmak.

Gereç ve yöntemler: Toplamda 30 Sprague-Dawley rat çalışmaya dahil edilip 3 eşit gruba ayrıldı. Birinci grup herhangi bir tedavi verilmeyen tamamen normal kontrol grubunu oluşturdu. Kalan ratların hepsine 60 mg/kg streptozosin (STZ) enjeksiyonu uygulanarak diyabetik olmaları sağlandı. Diyabetik sıçanlar ise iki eşit gruba ayrılarak diyabetik kontrol (DM kontrol) ve ESWT ile tedavi grubunu (DM+LESWT) oluşturdu. Çalışma için sıçan penis dokusu örneklerinde Endotelyal Nitrik Oksit Sentaz (eNOS) ve Vasküler Endotelyal Büyüme Faktörü (VEGF) mikro Ribo Nükleik Asit (mRNA) ekpresyon seviyeleri değerlendirilmiştir.

Bulgular: STZ dozundan sonra diyabetik kontrol grupta normal kontrol grubuna göre eNOS seviyesi düşerken, LESWT+DM grubunda eNOS seviyesinde istatistiksel olarak anlamlı artış olduğu görülmüştür. VEGF seviyesi de diyabetik kontrol grupta aynı şekilde STZ ile düşerken LESWT+DM grupta ise neredeyse başlangıç seviyelerine geldiği görülmüştür. Fakat VEGF değişimlerinin istatistiksel olarak anlamlı olmadığı saptanmıştır.

Sonuç: LESWT etki mekanizmasının penis dokusundaki etkisininin anjiyogenez faktörleri üzerinden olabileceği görülmektedir.

Anahtar Kelimeler: Anjiyogenez; hayvan deneyi; penis; şok dalga tedavisi.

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Introduction

Diabetes is a well-known risk factor for erectile dysfunction (ED). In fact, the prevalence rate of ED is 3 times higher (35-90%) among diabetic patients, compared with non-diabetic ones.[1,2] Although phosphodiesterase type 5 (PDE5) inhibitors are considered as the first line treatment modalities of ED, the response rate of diabetic patients to those medications is so low that only half of the patients benefit from this treatment.[3]

The etiology of diabetes-associated ED is multifactorial, including impaired vascular structure and endothelial dysfunction. In diabetic patients, endothelium-dependent impaired vasodilation is found to be associated with hyperglycemia as a result of endothelial nitric oxide synthase (eNOS) deficiency.[4] Using animal model, Bivalacqua et al.[5] found that the level of eNOS expression, nitric oxide (NO) and erectile response were also reduced following streptozotocin (STZ) treatment.[5]

Recent studies[6-8] have shown the potential therapeutic effect of low-energy shock wave therapy (LESWT) on organic ED. Post-treatment alterations in penile tissue, however, need further elucidation. Vascular improvement due to the stimulation of angiogenic factors on bone and cardiac tissues have been demonstrated in animal models. In a relevant study, Wang et al.[9] displayed the enhancing effect of LESWT on the expression of angiogenesis-related growth factors including eNOS and vascular endothelial growth factor (VEGF) in tendon-bone junction. Similarly, LESWT improved regional myocardial blood flow and the wall thickening fraction, and ensured complete recovery of the left ventricular (LV) ejection fraction in ischemic cardiac tissue.[10]

Therefore, the objective of this study is to assess the effect of LESWT on the angiogenesis in penile tissue in a STZ-induced diabetic rat model.

Material and methods

Study animals

The study was approved by Institutional Animal Care and Use Committee. Thirty Sprague-Dawley rats at six weeks of age were used as subjects. Whereas randomly selected 10 rats served as the control group (N), the remaining 20 rats received 60 mg/kg streptozotocin (STZ) through intraperitoneal route and their blood glucose levels were monitored weekly using the venous blood samples drawn from their tails. Rats with fasting blood glucose of >200 mg/dL were randomly divided into two different groups: Diabetic group (DM) and treatment group (DM+LESWT).

Shock wave treatment

Six weeks after the STZ injection, rats in the DM+LESWT group was exposed to LESWT Omnispec Extracorporeal Shock Wave Therapy System, ED1000TM (Medispec Ltd- Germantown, USA). An applicator tip with a diameter of 6 mm was used in order to deliver shock waves to the penile tissue more easily (Figure 1).

Anaesthetized rats were then placed in supine position and their prepuce was retracted. After application of ultrasound gel onto the penis, a shockwave applicator was placed and a total of 300 shocks were delivered at energy level of 0.1 mJ/mm^2 and frequency of 120 per minute. This procedure was repeated three times a week for a period of 2 weeks (Figure 2). At end of the 8th week, all rats used in the study were sacrificed.

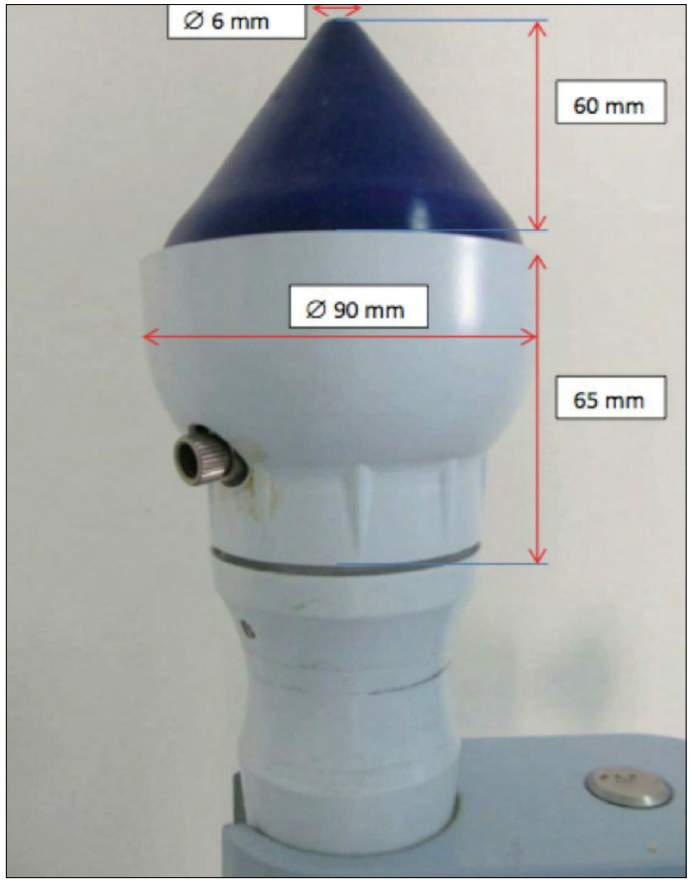

Figure 1. Applicator of the energy shock wave therapy machine

Total RNA extraction

After the shockwave therapy, penises of twenty-nine rats were stabilized in a RNA stabilisation reagent (vQiagen, Hilden, Germany). Tissue samples were then placed onto a Petri dish, then they were dissected into small pieces by surgical blades. Afterwards, the tissues were homogenized by using 0.1 mm diameter zirconia/silica beads (BioSpecProducts, Bartlesville, USA). RNA (High Pure RNA Tissue Kit, RocheDiagnostics, Mannheim, Germany) was isolated according to the manufacturer's instructions. RNA concentration and purity were determined by using NanoDrop 2000 C spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Reverse transcription

Following the extraction of RNA, cDNA synthesis was maintained with 100 ng per 1 μL RNA according to the manufacturer's instructions. (Easy Script Plus™ cDNA Synthesis Kit, Applied Biological Materials, Richmond, Canada) Primer Design Gene sequence information was obtained from the Entrez Nucleotide data base. Gene-specific primer sequences were obtained from "Primer Blast" tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and checked by "OligoAnalyzer 3.1" (Integrated DNA Technologies, Coralville, IA). GAPDH was selected as a housekeeping gene to normalize VEGFA and eNOS gene expressions.

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Real time PCR

cDNA samples were amplified using ECO Real Time PCR Instrument (Illumina, San Diego, CA, USA) with primer pairs designed to reflect the expression patterns of VEGFA, eNOS3 and GAPDH. Master mixes were prepared according to the manufacturer's protocol of ABM Eva Green qPCR Mastermix-low ROX (Applied Biological Materials, Richmond, Canada). Total reaction volume was 20 μL including 1 μL of cDNA sample. Blank samples without template DNA were administrated to ensure that there was no contamination inside the wells.

RT-PCR amplification was performed under following temperature conditions,and for indicated periods of time: cycle 1:95°C/10 min; cycle 2:95°C/30s, 58°C/1 min (40x); cycle 3: 95°C/15 s, 55°C for 15 s, and 95°C/15 s. The relative quantification method was used and the results were monitored by Eco Study Software (Illumina, San Diego, CA, USA).

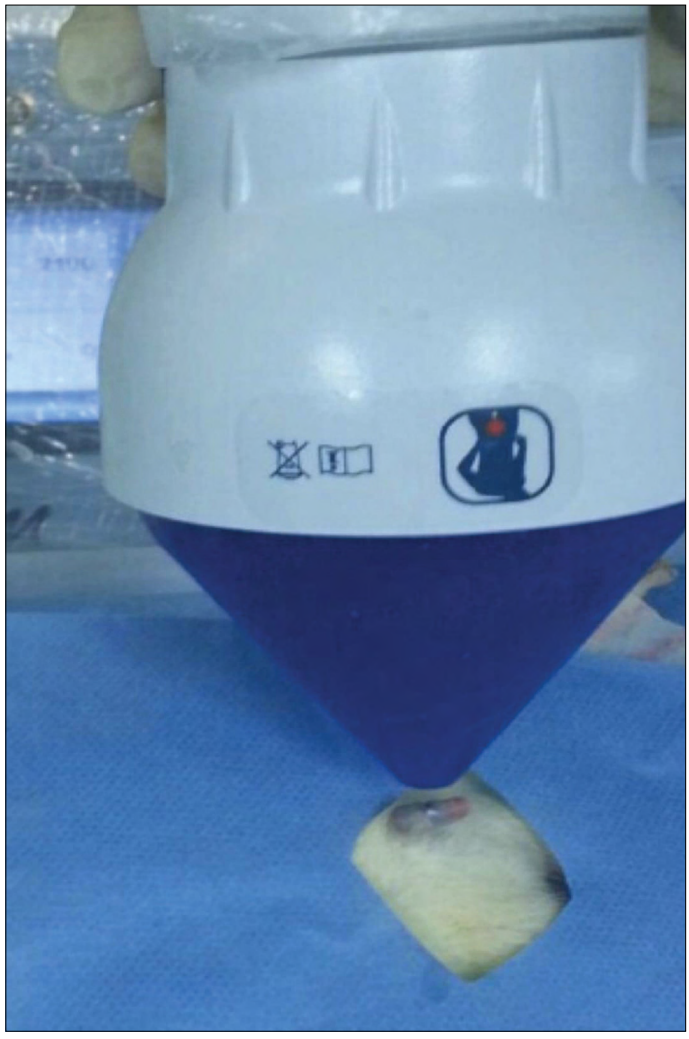

Figure 2. Shockwave application to the rat penis

The molecular effect of LESWT on penile tissue was evaluated via the measurement of alteration in eNOS and VEGF mRNA expressions. Data were analyzed using a One Way ANOVA test (post hoc Tukey). Statistical significance was set at $p<0.05$.

Results

While eNOS expression level was significantly lower in the DM group compared to the controls (1 vs. 0.56 relative quantification (rq) $p<0.05$), it was attenuated in the LESWT group. (0.56 vs. 0.85 rq, $p<0.05$). The eNOS level of the control group was still higher than the treatment group, but intergroup difference was not statistically significant (0.85 vs. 1 rq, $p>0.05$) (Figure 3).

VEGF expression levels in penile tissue were slightly reduced in the diabetic group compared with the control group without any statistically significant intergroup difference (1 vs. 0.82 rq $p>0.05$). Similar to eNOS expression, VEGF expression level was also improved after LESWT; although the effect was not statistically significant (1 vs. 1.04 rq $p>0.05$) (Figure 4).

Discussion

Erectile dysfunction treatment in diabetic patients is still a challenge despite the available treatment modalities such as PDE-5 inhibitors, intracavernosal injection and vacuum erection devices. On demand usage of PDE-5 inhibitors undeniably revolutionized the ED treatment and became the first-line treatment option for most of the patients.[11] The efficacy rates of PDE-5 inhibitors however, are lower in diabetic ED patients and there is no evidence suggesting PDE-5 inhibitors can reverse the pathological changes and thus provide a permanent solution in the presence of diabetes.[12]

Recently, some promising novel treatment modalities came into prominence as new solutions for DM- associated ED. One of

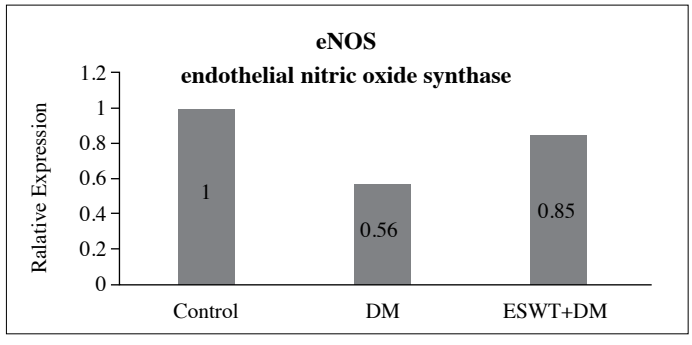

Figure 3. eNOS expression levels in the control, DM, and ESWT + DM groups

DM compared to the control :1 vs 0.56 rq, $p<0.05$
DM compared to ESWT+DM:0.56 vs 0.85 rq, $p<0.05$
ESWT: energy shockwave therapy; DM: diabetes mellitus

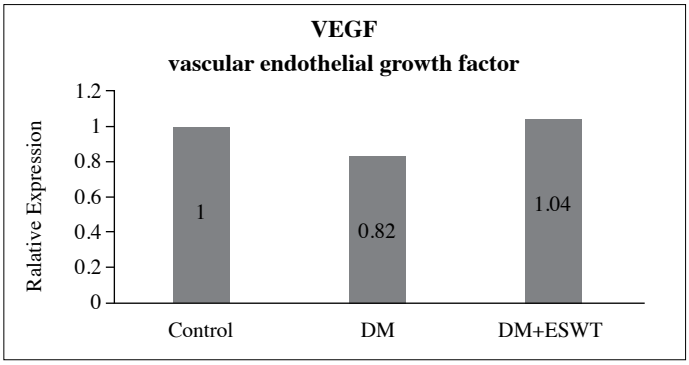

Figure 4. VEGF expression levels in the control, DM, and ESWT + DM groups

p>0.05, Not significant between all groups
ESWT: energy shockwave therapy; DM: diabetes mellitus; VEGF: vascular endothelial growth factor

those, energy shock wave therapy (ESWT) was reported to improve the local blood supply in several different animal studies.[13] Also in clinical trials, low-energy shock wave therapy (L-ESWT) treatment improved the erectile capacity of patients with organic ED and/or severe ED refractory to PDE-5 inhibitors treatment.[14]

However, pathophysiological mechanism of the effect of ESWT on ED and penis still lacks a well-defined explanation. The current hypothesis suggests a neo-angiogenesis at penile tissue that underlies-ESWT treatment mechanism: There are a few animal studies where the effects of ESWT on angiogenetic factors including VEGF, PCNA and NOS levels were investigated.[15]

Nitric oxide and VEGF have been shown to be important mediators of angiogenesis in these studies, and it has been suggested that the mechanism of shock wave therapy involves the early release of angiogenesis growth factors (eNOS and VEGF) and subsequent induction of neovascularization and tissue proliferation.[16-18] Wang et al.[9] demonstrated this angiogenesis inducing effect of shock wave therapy on the tendon–bone junction of rabbits, which successfully resulted in neo-vessel growth and tissue proliferation.

In our study, eNOS and VEGF levels of the STZ induced diabetic rats were assessed following ESWT treatment. According to our results, ESWT treatment of diabetic rats significantly restores the blunted eNOS levels and thus ameliorates the deteriorating effect of diabetes on penile tissue. The protocol of our study, which was successfully applied in cardiology before, was designed based on the work of Vardi et al.[7], which achieved neovascularization for erectile dysfunction in humans. VEGF is another key regulator of physiological angiogenesis.[19] It has been proved that L-ESWT treatment of human myocardial tissue undergoing ischemia upregulates the expression of VEGF and induces neovascularization devoid of any adverse effects.[19] A similar restorative effect of L-EWST on the VEGF expression levels was observed in our diabetic rats without any statistical significance.

Expression levels of VEGF mRNA in the DM-SW group were almost identical to control groups. In rats with prolonged diabetes, and thus exposed to more pronounced effects of DM, the positive recovery effect of ESWT may reach statistical significance. This notion is supported by Liu et al.[20] study in which expression levels of VEGF significantly changed in diabetic rats which received ESWT treatment twelve weeks after the STZ injection.[20]

Ito et al.[21] reported the effectiveness of L-ESWT treatment on cardiovascular diseases via promotion of angiogenesis through upregulation of the expression levels of related molecules levels including VEGF. After the demonstration of microcirculation augmenting effect L-ESWT, the center of interest has gradually shifted from shock waves to L-ESWT.

There are two animal studies examining the effect of ESWT on penile tissue. In the first relevant study, Liu et al.[20] investigated the therapeutic effect of LESWT at different doses on STZ-induced diabetic rats with ED. In this study, L-ESWT treatment was performed three times a week for two weeks. The assessment of erectile function via measurements of intracavernous pressures, indicated an improvement in diabetic rats which underwent L-ESW therapy.

While Liu et al.[20] observed an increase in the smooth muscle and endothelial content of corpus cavernosum following LESWT treatment, they reported a concomitant upregulation of alpha smooth muscle actin (α-SMA), *Von Willebrand* factor (vWF) nNOS, and VEGF levels and downregulation of the RAGE expression levels.[20]

In a similar protocol to our work, second study was published by Qiu et al.[22] with an objective of investigating the effects of ESWT on three different groups of rats (diabetic control, normal control and ESWT- treated diabetic rat groups). In this study, endogenous mesenchymal stem cells were tracked using 5-ethynyl-2 deoxyuridine marker. Treatment procedure was administered three times a week for 2 weeks. The results have demonstrated that LESWT could partially ameliorate DM-associated ED by promoting regeneration of smooth muscle, endothelium, and nNOS-positive nerves.[22]

Several limitations must be considered when interpreting these findings. Firstly, there is no data about erectile function in rats before and after the shock wave treatment. Secondly, the histological alterations in penis architecture after shock wave therapy is unknown.

Mechanism action of ESWT on the penis retains its enigmatic nature. A potential explanation was related to neo-angiogenetic influence of this treatment on the penile tissue. The ameliorating effect of ESWT on decreased eNOS and VEGF expression levels in diabetic rats supports this hypothesis. Increased vascularization of penile tissue might lead to the regeneration of endothelial and smooth muscle tissue and thus explains the mechanism of recovery of the erectile function after ESWT treatment.

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The near identical levels of eNOS and VEGF in ESWT treated diabetic rats and normal controls promise a pronounced remedy of DM- associated erectile dysfunction. To our knowledge, this is the first study evaluating eNOS levels in ESWT treated diabetic rat penile tissue. This study should be further supported by additional studies to fully elucidate ESWT mechanism of action.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of İstanbul University Ethics Committes for Animal Studies (No:2013/46).

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