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Original Article

Mechanism research of improvement of diabetic ED rats' erectile function by vacuum erection device through anti-oxidative stress

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Abstract: Objective: To investigate whether the vacuum erection device (VED) therapy improves the erectile function through anti oxidative stress injury. Methods: Adult male sprague-dawley (SD) rats (n=35) were randomly divided into normal control group (n=8) and Type 2 diabetic model group (n=27). Eight weeks later, 17 diabetic erectile dysfunction (ED) rats that were selected through apolipoprotein (APO) test were randomly divided into ED group (n=8), and ED+VED group (n=9). One month after the VED treatment, erectile function was assessed by measuring the rise in intracavernous pressure (ICP) of the rats following cavernous nerve electrostimulation before the rats were sacrificed. The activities of superoxide dismutase (SOD) and the level of malondialdehyde (MDA) in cavernous tissues were detected. The Hematoxylin-eosin (HE), Masson and terminal deoxynucleotidyl transferase biotin-dUTPnick end labeling (TUNEL) staining were observed under light microscope. Immunohistochemistry and western blot (WB) were used to assess the expression of endothelial nitric oxide synthase (eNOS), protein kinase B1 (AKT1), phosphorylated protein kinase B (p-Akt); hypoxia inducible factor-1α (HIF-1α), NADPH oxidases 2 (Nox2), NADPH oxidases 4 (Nox4). Results: (1) Compared with ED group, a significant increase in ICP/MAP was recorded in the ED+VED group (0.42±0.09 VS. 0.21±0.07), P<0.05, but it is still not up to the level of the control group (0.73±0.10 VS. 0.42±0.09), P<0.05. (2) Compared with ED group, the activities of SOD in the ED+VED group increased significantly (80.65±4.60 VS. 59.80±3.20), P<0.05 and the levels of MDA decreased significantly (4.39±0.44 VS. 6.49±0.48), P<0.05 in cavernous tissues. But it is still not up to the level of the control group. (3) Compared with ED group, the penile vascular smooth muscle increased, collagenous fiber decreased and smooth muscle/collagen proportion ratio increased in the ED+VED group (0.31±0.02) VS. (0.25±0.03), P<0.05. But it was inferior to the control group (0.62±0.03) VS. (0.31±0.02), P<0.05. (4) Compared with ED group, the apoptosis rate decreased in the ED+VED group (60.1% VS. 31.3%), P<0.05, but it exceeded the control group (24.6% VS. 31.3%), P<0.05. (5) Compared with ED group, the expressions of HIF-1α, Nox2, Nox4 were decreased while p-AkT, AKT1, eNOS were increased in the ED+VED group. But it is still not up to the level of the control group. Conclusion: Vacuum erection device can improve erectile function of diabetic ED rats through anti-oxidative stress injury. The mechanism might be that VED improves hypoxia state of penile tissues, decreases Nox/ROS oxidase pathway, and enhances the eNOS/Akt pathway, which leads to penile vascular endothelial cells and vascular smooth muscle cells repairing, as well as cell apoptosis decreasing.

Keywords: Diabetes mellitus, erectile dysfunction, vacuum erection device, oxidative stress

Introduction

Diabetic erectile dysfunction (DED) is a kind of refractory ED and the effective rate of treatment with first-line PDE5i alone is only 50% [1, 2]. As a noninvasive and effective treatment, vacuum erection device (VED) is one of effective alternative and adjuvant treatments for DED which is not responding to PDE5i alone.

In our early studies, we found combined use of sildenafil and vacuum erection device therapy

significantly enhanced erectile function, and it is well tolerated by DED patients not responding to first-line sildenafil alone. We also found the mechanism might be the VED can improve the cavernosum arteries blood flow shear stress in DED rats corpus, inhibit the apoptosis of smooth cells and endothelial cells in the corpus cavernosum, as well as increase the expression of endothelial nitricoxide synthase (eNOS) in the corpus cavernosum endothelial cells (CCECs) [3, 4]. However, the mechanism why VED can improve erectile function remains unclear.

Oxidative stress injury is one of the important mechanisms of the occurrence and development of erectile dysfunction. There are several evidences showed that erectile function can be improved through the anti-oxidative stress injury. Therefore, in this study, DED rats were treated with VED, and then the changes of erectile function, ROS, eNOS/Akt signal pathway were evaluated. We hope to further clarify whether the VED therapy improves the erectile function through anti oxidative stress injury.

Materials and methods

Animal grouping and VED treatment

All animal experiments were approved by The Third Affiliated Hospital of Sun Yat-sen University Animal Care and Use Committee, in accordance with public health service guidelines. For this study, thirty-five male SD rats (180-220 g) were obtained from Guangdong Medical Laboratory Animal Center and were randomly divided into normal control group (n=8) and Type 2 diabetic model group (n=27), and the same method as the previous reports was used [5]. Eight weeks later, 17 diabetic ED rats that were selected through APO test (same as the previous reports [6]) were randomly divided into ED group (n=8); ED+VED group (n=9). ED+VED group were treated with VED in a month. The parameter was that the 20 kpa of negative pressure, 1.67 kHz of frequency, 0.4 s of pulse width, 30 min of treatment time, which were detected every two days, with a total of 15 times. One month after the treatment, measurements of the intracavernous pressure (ICP) and the ratio of ICP/mean arterial pressure (MAP) were used to assess erectile function. The ICP measurement, ICP/MAP calculation, and the method of cavernosal nerve stimulation have been described previously [7]. The animals were then sacrificed and the penises were removed and cleaned. Each group harvested penile tissues of 4 rats to detect the activities of SOD and the level of MDA. The rest of rat penis were also collected. Part of the proximal penises were fixed in 4% paraformaldehyde and the remainder was stored in refrigerator at -80°C for western blotting.

Biochemical markers of oxidative stress

The superoxide dismutase (SOD) activity and malondialdehyde (MDA) levels were used to assess oxidative stress. The SOD and MDA levels in the corpus cavernosum was detected as described previously [8]. All assay kits were purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China) [7].

Histopathology

Following routine dehydration and paraffin embedding, tissue samples were cut into 5mm sections from the midshaft of the penis mounted on slides and dried. Then the tissue slides, showing the cross-section of the corpora cavernosa (CC), were deparaffinized and rehydrated for following studies.

HE staining

To assess the morphological structure of the rat penile tissues and all operations were strictly performed according to the kit instructions (Nanjing Jiancheng Bioengineering Institute).

Masson's trichrome stain

To assess the smooth muscle/collagen ratio, slides were stained for Masson's trichrome (MT) according to standard protocol, which was reported previously [7]. The nuclei stained blue, the smooth muscle stained red, and the collagen stained green. The Smooth muscle/collagen ratios were analyzed by using Image Pro Plus 6.0 software (Media Cybernetics, Inc., Bethesda, MD, USA). One slide per animal (slides were from the midshaft of penis about the same level) was used to calculate the ratio of the red-staining smooth muscle to the blue-staining collagen content in the cross-section of the CC building of the group average. The ratios were compared among the three groups.

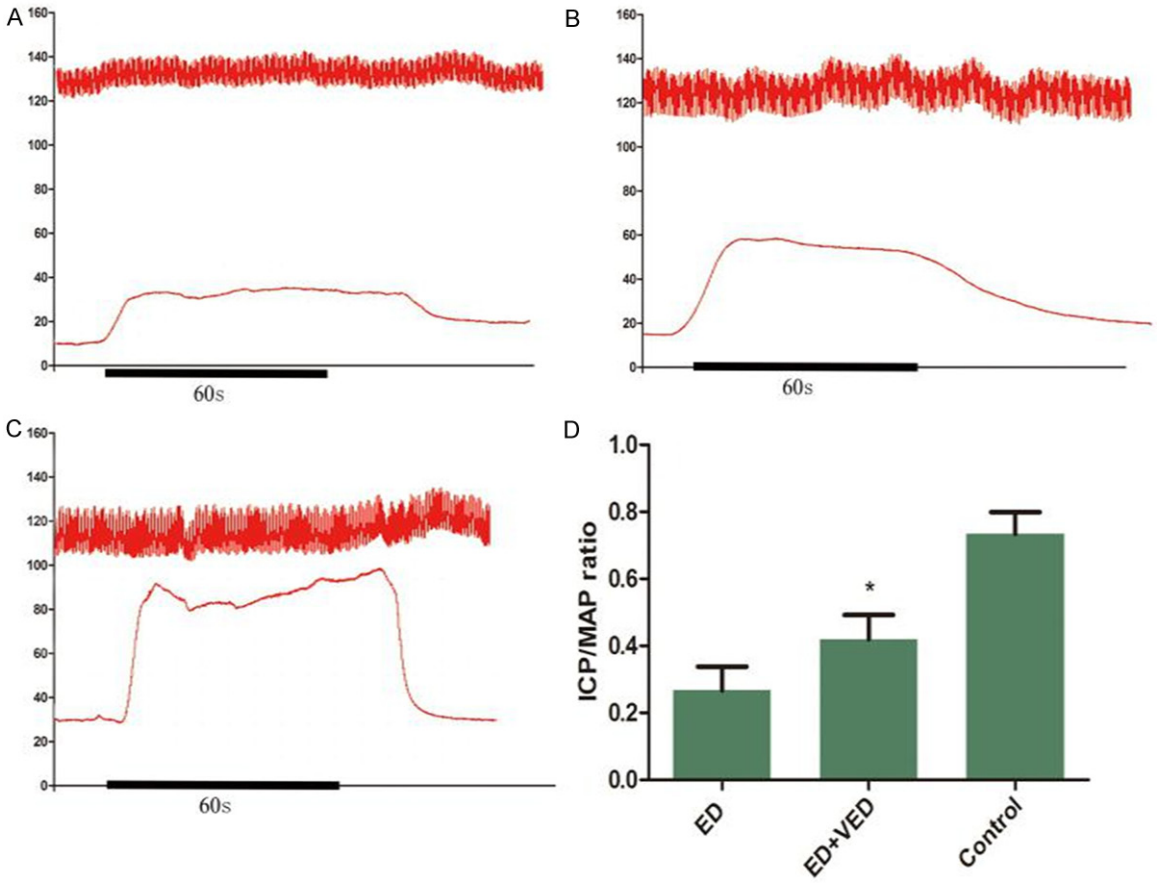

Figure 1. Erectile function evaluation. Peak intracavernous pressure (ICP)/mean arterial pressure (MAP). A. ED group. B. ED+VED group. C. Control group. D. The chart of ICP/MAP ratio. Peak ICP/MAP values declined significantly in ED group. VED therapy could partially regain the reduction, but could not bring erectile function to normal level. *P<0.05 vs. control group; P<0.05 vs. ED group.

Apoptosis assessment

To evaluate apoptosis, the terminal deoxy nucleotidyl transferase biotin-d UTP nick end labeling (TUNEL) assay was executed following the manufacturer's instructions (Roche Applied Science, Mannheim, Germany). Three slides from three different animals per group were selected randomly. Every slide was analyzed by counting cells in six non-overlapping zones of the entire section at × 400 magnification. The number of TUNEL cells was expressed as a percent of the total number of cells and reported as the apoptotic index (AI).

Immunohistochemistry

For immunohistochemistry, tissues were fixed in 4% paraformaldehyde overnight. Following deparaffinization and rehydration, sections (5 mm) were rinsed for 10 min by using phosphate-buffered solution. Endogenous peroxidase activity was quenched by using 3% H_2O_2 for 10 min. After 10-min washing by phosphate-buffered solution three times, tissues were incubated with antibodies targeted against eNOS (Abcam, USA; 1:1000), AKT1 (Abcam, USA; 1:1000), p-Akt (Abcam, USA; 1:300); HIF-1 alpha (Abcam, USA; 1:200), Nox2 (Abcam, USA; 1:200), Nox4 (Abcam, USA; 1:200) at 4°C overnight. Sections were then incubated with biotinylatedanti-mouse or anti-rabbit secondary antibodies (Abcam, USA, China; 1:200) for 30 min at 37°C and then counterstained with hematoxylin. Sections incubated without primary antibodies were used as negative controls. Images were captured by Nikon microscope--a Spot RT color digital camera [9].

Western blot analysis

Western blotting was used to measure the protein expression of eNOS, AKT1, p-Akt; HIF-1α,

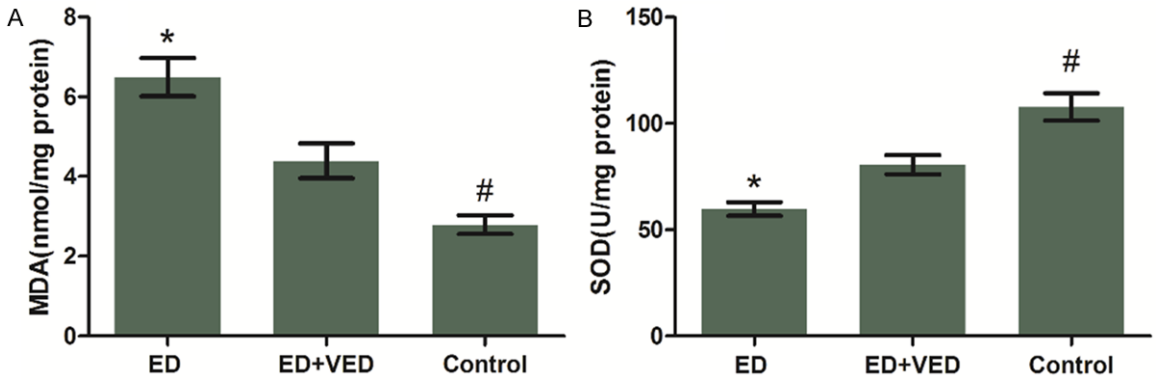

Figure 2. MDA and SOD activity in the cavernosum. Malondialdehyde (MDA) levels: (A) The level of MDA in the corpus cavernosum of ED+VED group was lower than that in ED group (P<0.05). Superoxide dismutase (SOD) activity (B) the penile SOD activity was significantly higher in ED+VED group compared with ED group (P<0.05). But they were still not up to the level of the control group. All the data are expressed as mean ± sd. *P<0.05 vs. ED group; #P<0.05 vs. control group.

Nox2, Nox4 in the cavernosum. The penile tissues that had been stored in refrigerator at -80°C and lysed in Tissue Total Protein Lysis Buffer. The samples were then grounded on ice and centrifuged at 12000 rpm for 12 min at 4°C. The supernatants were assembled and stored at -80°C. Equal amounts of proteins were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and then transferred to a nitrocellulose membrane. The membrane was then incubated with antibodies targeted against eNOS (Abcam, USA; 1:1000), AKT1 (Abcam, USA; 1:5000), p-Akt (Abcam, USA; 1:500); HIF-1alpha (Abcam, USA; 1:200), Nox2 (Abcam USA; 1:1000), Nox4 (Abcam USA; 1:500) or GAPDH (Abcam USA; 1:1000) at 4°C overnight and then for 1 h at room temperature with anti-mouse (Abcam, USA; 1:5000) or anti-rabbit (Abcam, USA; 1:5000) secondary antibodies. Detection was performed by adopting enhanced chemiluminescence followed by autoradiography [10].

Statistical analysis

All statistics were completed by the statistical software SPSS.20. P<0.05 was considered as statistically significant. All data represented the results of more than 3 repeated experiments, and were expressed as mean ± sd. The comparison of 3 groups of continuous variables were analyzed by one-way ANOVA. Among them, homogeneity of variance was multiple compared by LSD, and heterogeneity of variance was multiple compared by Dunnett's T3.

Results

Erectile function evaluation

The ICP/MAP ratio was shown in **Figure 1**. When compared with control group, the mean ICP/MAP ratio in ED group declined significantly (P<0.05). After the VED treatment for onemonth, the mean ICP/MAP ratio in ED+VED group elevated (P<0.05). Thus, VED treatment could improve the erectile function of DED rats according to ICP/MAP ratio (**Figure 1**).

Biochemical markers of oxidative stress

The MDA levels and SOD activity in the penial tissues were shown in **Figure 2**. Increased MDA and decreased SOD activity levels were found in the penial tissues of ED group compared with control group (P<0.05). Following treatment with VED, MDA levels decreased and SOD activity increased (P<0.05).

Histopathology

HE staining: HE staining showed that control group has a large number of irregular blood sinus clearance which attached to continuous vascular smooth muscle (**Figure 3C**). ED group saw fewer irregular blood sinus clearance which attached to in continuous vascular smooth muscle (**Figure 3A**). One month after VED therapy, the smooth muscle content was significantly increased and collagen content was decreased in the corpus cavernosum of ED+VED group (**Figure 3B**).

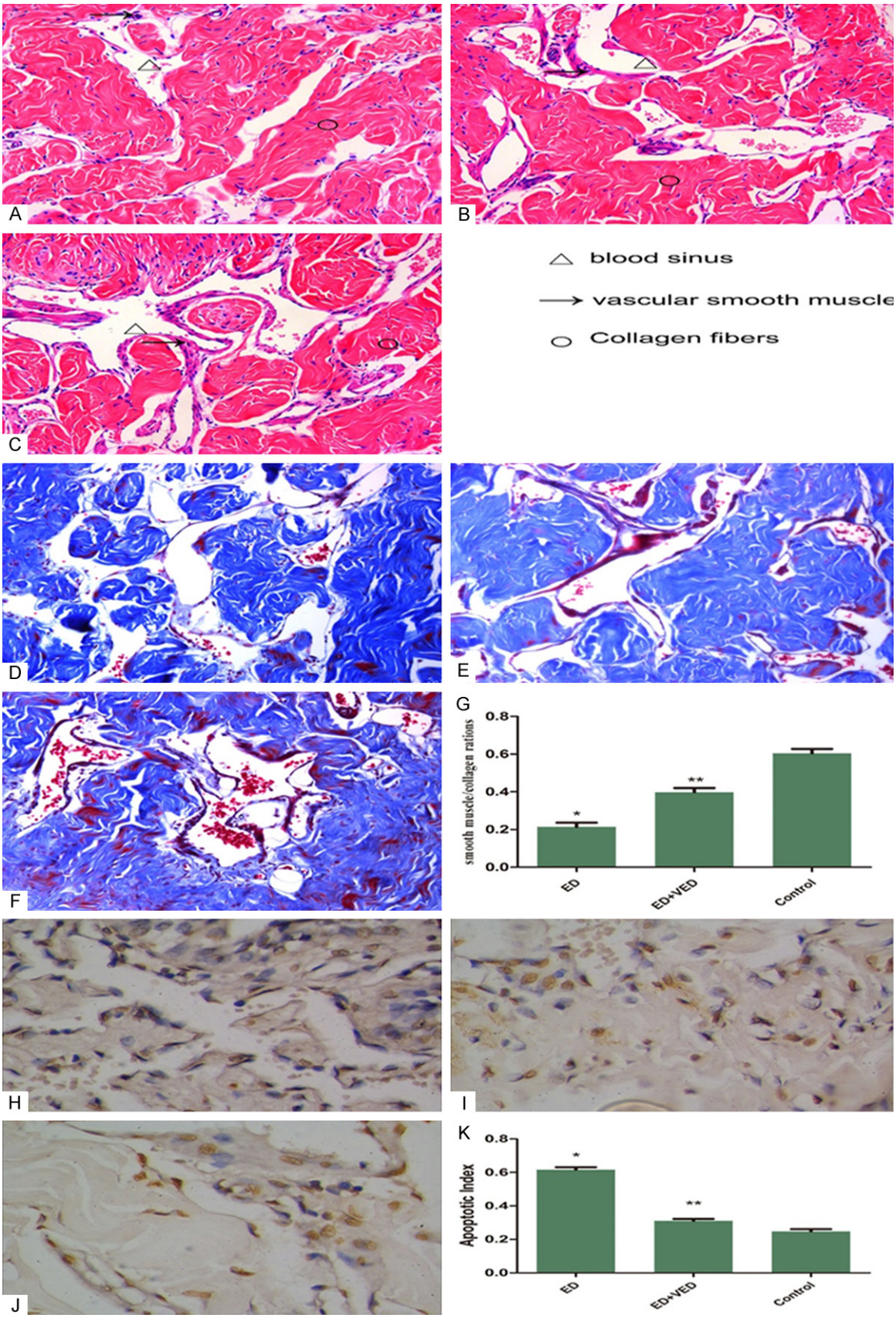
A
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Figure 3. (A-C) HE staining of cavernosum tissue. Collagen fibrils in the penis were apparently damaged and lost their characteristic undulating appearances. The endothelial and smooth muscle cells were also degenerated and interstitial cells proliferated. Original magnification × 200. HE staining of cavernous tissues of rats in the ED group (A), ED+VED group (B) and control group (C). (D-G) Masson's trichome staining of cavernosum tissues. The VED therapy was conducive to the restoration of penile tissue structure impairment in ED+VED group. (D) ED group; (E) ED+VED group; (F) Control group; (G) The chart of smooth muscle/collagen ratio; (H-K) TUNEL assessment of apoptosis. A reduced apoptotic indices (AI) percentage for VED therapy was found compared with ED group ($P<0.05$), although still higher than that of the control group ($P<0.05$). (H) ED group; (I) ED+VED group; (J) Control group; (K) The chart of AI ratio. *$P<0.05$ vs. ED group; $P<0.05$ vs. control group.

Smooth muscle/collagen ratios: The Masson's trichrome staining in the Control group showed smooth muscle/collagen ratios of 0.62±0.03, the supreme smooth muscle/collagen ratio of three groups (**Figure 3F**). This result was significantly higher compared with ED group (0.62± 0.03 VS. 0.25±0.03, $P<0.05$) (**Figure 3D**) and remained superior to the ED+VED group as well (0.62±0.03 VS. 0.31±0.02) (**Figure 3E**). The ED+VED group was significantly increased compared with the ED group (0.31±0.02 VS. 0.25±0.03, $P<0.05$), and revealed an obvious trend toward improvement.

Apoptosis analysis: In **Figure 3H-K**, we displayed that the DED rats treated with VED indicated a significant reduction in apoptotic indices (AI) within the penial tissues compared with ED group (31.3% VS. 60.1%, $P<0.05$) (**Figure 3H**, **3I**). By contrast, the AI value in the control group was 24.6% (**Figure 3J**), which was lower than the ED+VED group (24.6% *vs.* 31.3%, $P<0.05$).

Protein levels of eNOS, AKT1, p-Akt; HIF-1α, Nox2, Nox4 in cavernous tissue

Immunohistochemistry: Immunohistochemical staining of endothelial nitric oxide synthase (eNOS) in the ED group (**Figure 4A**) was declined compared with the control group (**Figure 4C**). The eNOS staining in the ED+VED group (**Figure 4B**) was increased compared with ED group; however, it was still lower than that of control group. The expression of AKT1, p-AKT revealed the same trend (**Figure 4D-I**).

Immunohistochemical staining of HIF-1α in the ED group (**Figure 4J**) was elevated compared with the control group (**Figure 4L**). The ED+VED treatment was partially reversed (**Figure 4K**), despite it was still higher than control group. The expression of Nox2, Nox4 revealed the same trend (**Figure 4M-R**).

Western blot analysis: As showed in **Figure 4S**, the expression of eNOS, AKT1, p-Akt protein declined in the ED group when compared with control group, but the expression of HIF-1α, Nox2, Nox4 elevated. After VED treatment, HIF-1α, Nox2, Nox4 proteins declined. On the contrary, the expression of eNOS, AKT1, p-Akt elevated in the ED+VED group when compared with the ED group that were not treated.

Discussion

HIF-1α is the key factor to maintain the oxygen balance in vivo [11], as a transcription factor, it mediates hypoxia response by inducing a large number of gene expression that associated with hypoxia responses [12]. Under normal conditions, HIF-1α is easy to decompose with a half-life of 10 minutes [13]. Under the condition of hypoxia, however, the proline and the ugly amine residues have not been hydroxylation, which can prevent the hydrolysis of the protein. This may realize the HIF-1α transfermation from intracellular to extracellular. Previous studies indicated that the HIF-1α expression level can be maintained for 14 days under the condition of continuous hypoxia [14].

Oxidative stress refers to a series of pathophysiological reactions produced by the accumulation of various kinds of free radicals such as reactive oxygen species (ROS). Brownlee [15] et al. thought that oxidative stress throughout the whole process of diabetes and oxidative stress is also still the common basis of the pathogenesis of diabetic complications. Paneni et al. [16] pointed out that the imbalance between ROS accumulation triggered by hyperglycemia and nitric oxide (NO) was the key to the endothelial dysfunction. Under the impact of a large number of ROS in the cell, the intracellular balance between the Ca^{2+} level, ROS and the content of ATP would be broken. A large number of ROS and high level of Ca^{2+} could cause the decline of mitochondrial membrane potential

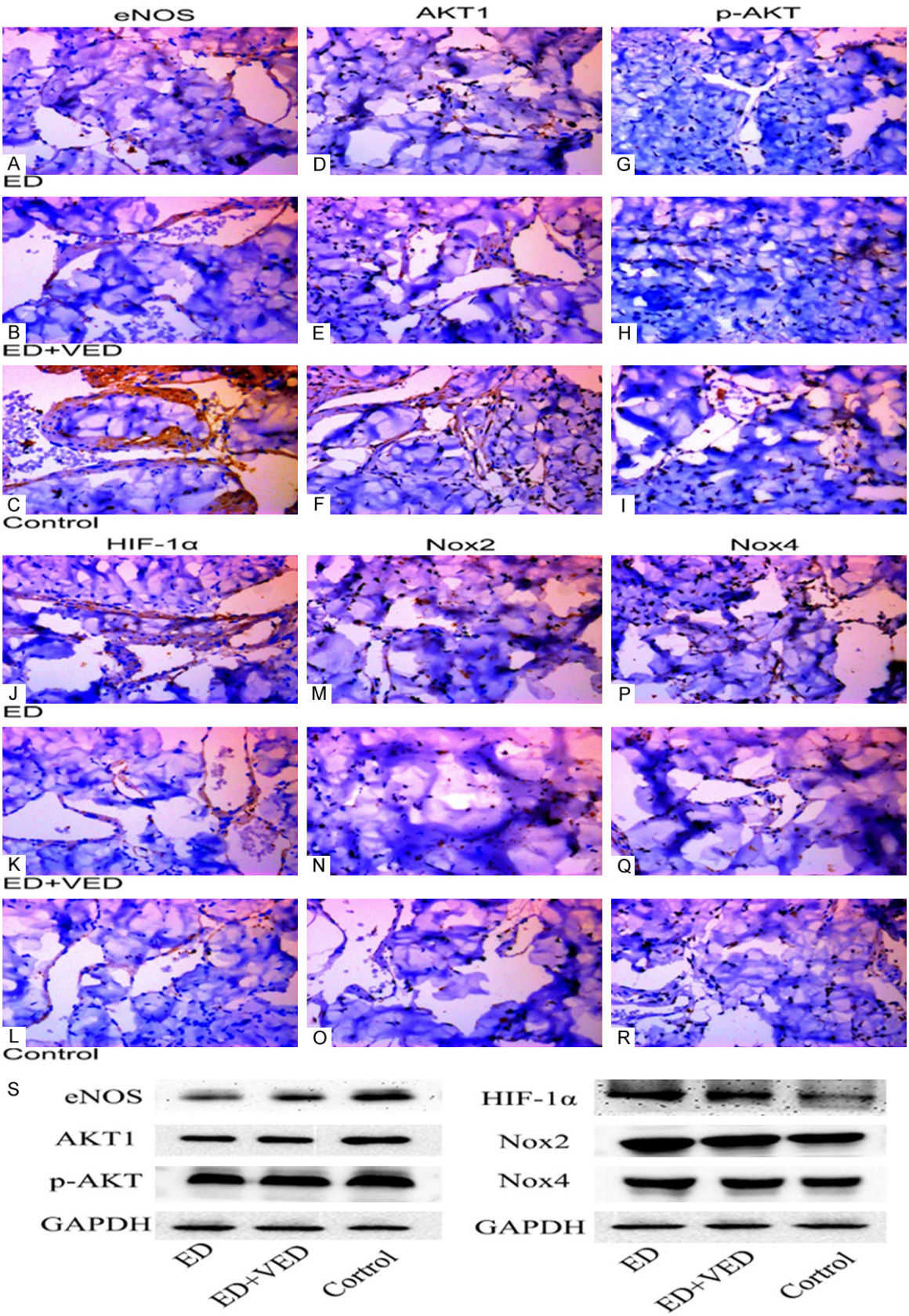
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Figure 4. (A-R) Are immunohistochemical staining of eNOS, AKT1, p-Akt; HIF-1α, Nox2, Nox4. Compared with the control group, vacuum erectile device (VED) therapy improved penile eNOS AKT1, p-Akt expression (A-I), and reduced transforming HIF-1α, Nox2, Nox4 expression (J-R). But could not bring them to normal level. (S) Is the protein level of eNOS, AKT1, p-Akt; HIF-1α, Nox2, Nox4 in penile tissues. eNOS, AKT1, p-Akt protein declined in ED group and elevated in ED+VED group. Compared with ED+VED group, the HIF-1α, Nox2, Nox4 protein elevated in the ED group. But they were still not up to the level of the control group.

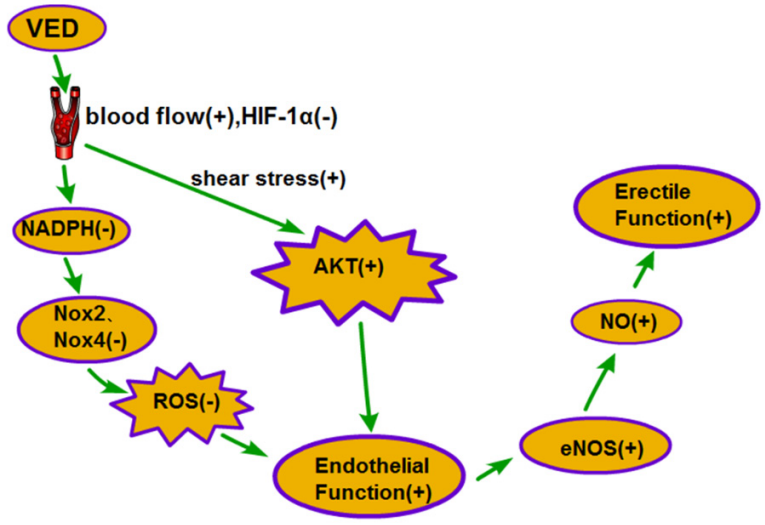

Figure 5. Possible mechanisms of VED improving erectile function.VED: vacuum erection device; NADPH: nicotinamide adenine dinucleotide phosphate; Nox2: NADPH oxidases 2; Nox4: NADPH oxidases 4; ROS: reactive oxygen species; eNOS: endothelial nitric oxide synthase; NO: nitric oxide.

and then the ATP synthesis was inhibited significantly. What's worse, the change of permeability of mitochondria leaded to cell swelling, rupture and death. Therefore, oxidative stress can damage corpus cavernosum smooth muscle and lead to endothelial cell dysfunction and apoptosis, which may cause erectile dysfunction [17, 18].

More than 90% of ED patients could perform functional erectile after the VED therapy [2]. But its mechanism which improves erectile function is not clear. Previous studies suggested the mechanism of VED treatment was that the blood could be pumped into cavernous body by the negative pressure and then put narrow rings on the root of the penis to prevent venous reflux, which could maintain engorgement and erection as well. However, recent clinical studies have indicated that many ED patients have been performed VED without a narrow ring for treatment, which also can maintain an erection and promote the recovery of natural erection. This phenomenon cannot be explained in accordance with the previous theory. The main Mechanism of Treatment is as follows by Li Enchun et al. [10]. The VED could prevent penile venous blood outflow through the mechanical properties of the constriction ring. And penis cavernous nerve, muscle and blood vessels could be improved and stimulated by the negative pressure. As a result, the neurotransmitter-nitric oxide (NO) increased. Besides, the increased cavernous blood promoted the fluid shear stress, which contributed to endothelial nitric oxide synthase activation and increased the release of NO. NO was the vital messenger molecule in NO/cGMP signaling pathway contributing to the corpus cavernosum diastole [19, 20]. Then the tunica albuginea pressure could be increased by the diastolic penis. It helps to prevent the outflow of venous blood and maintain an erection [10].

We constructed the DED model, using VED to treat DED rats, and found that there was a significant improvement in erectile function after treatment of DED rats. At the same time, the activity of SOD in corpus cavernosum of rats in ED group was significantly lower than the rats in the ED+VED group ($P<0.05$) and the level of MDA increased significantly ($P<0.05$). SOD can resist and block the damage caused by intracellular oxygen radicals and repair the damaged cells in time, which play an important role in the oxidative balance of the body. MDA is the product of lipid peroxidation, which reflects the degree of oxidation in vivo. After VED treatment, the reduced levels of oxidative stress suggested that it play an important role in the improvement of erectile function. Considering with our previous studies, VED treatment can improve DED rats cavernosal artery blood flow shear stress, reduce the apoptosis rate of cor-

pus cavernosum cells, increase eNOS expression. We thought that the anti-oxidative stress may play an important role in the improvement of the erectile function of DED rats. In this regard, we analyzed the NADPH signaling pathway that produced ROS and Akt/eNOS signaling pathway. It was found that the expression of HIF-1, Nox2 and Nox4 decreased in the penile tissues of DED rats after VED therapy, while the expression of VED, AKT1, eNOS and p-Akt increased. So we suspected that the mechanism was that VED could increase perfusion and oxygen supply of the penis artery and reverse the penis hypoxia, which may lead the inhibition of HIF-1 alpha, Nox2 and Nox4. Nox4 and Nox2 are the main functional subunits of NADPH Oxidase which produces ROS in endothelial cells [21, 22]. Decreased expression of Nox2 and Nox4 could reduce the ROS and then reduce the oxidative stress response, which makes the endothelial cell function to be restored [21, 22]. At the same time, increased shear stress caused the expression of Akt/eNOS signaling pathway and enhance the release of NO, both of which promoted the improvement of penile erectile function (**Figure 5**).

Acknowledgements

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Disclosure of conflict of interest

None.

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