

## Effects of Selective Endothelin Receptor Antagonist on Vegf Signaling System in Diabetic Penis: A Possible Clinical Implication for Diabetes Induced Erectile Dysfunction

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### ABSTRACT

Erectile dysfunction (ED) affects around 50 percent of male diabetic patients due to vascular and neuropathic complications arising from diabetes. Vascular endothelial growth factor (VEGF) has been extensively investigated for its pathogenic relevance in various diabetes complications, and we have already noted that VEGF signaling is significantly reduced at the insulin-resistant stage in the penis of type II diabetic rat model. The present research used a three-week streptozotocin (STZ)-induced diabetic (DM) rat model to determine the expression of VEGF with NO mechanism in penile tissue and subsequently investigated its impact endothelin antagonism on these shifts. Citrate saline vehicle or STZ (65 mg/kg IP) was administered to male Sprague-Dawley rats (weight 453 ± 23 g). Hyperglycemia confirmed diabetes, and then after 1 week of diabetes, animals were divided into receiving endothelin-A (ET-A) receptor antagonists (TA-0201, 1 mg/kg) or saline by osmotic minipump for 2 weeks and then sacrificed. Significantly improved glucose levels in DM rats (405 ± 103 mg/dL) relative to non-DM rats (120 ± 8 mg/dL) and higher local ET-1 levels in DM penis by 20% are observed. A 30 percent decline in penile tissue expression of VEGF was observed in DM rats and improved by an antagonist with ET. Penile NO and eNOS levels in DM rats decreased, while ET-A receptor antagonist significantly increased. Therefore, we infer that ET antagonism seemed able to restore the down-regulated VEGF and was possibly efficient in restoring the decreased levels of NO and eNOS in DM.

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## Keywords

Erectile dysfunction, Diabetes, VEGF, Endothelin antagonism.

## Introduction

Erectile dysfunction (ED) happens in about 50 % of men suffering from diabetes mellitus (DM) [1]. The possible factor of ER in diabetic men can be the neuropathic and vascular complications arising from DM. Still, there can be multiple pathophysiological pathways through which ED occurs in diabetic patients [2]. These pathways can be endothelial dysfunction, neuropathy, hormonal changes, and cavernosal smooth muscle structural/functional changes [3,4]. These changes can occur in both types of diabetes; however, these are more prominent in type 1 DM [5,6]. Endothelial dysfunction is an essential component of diabetic ED [7]. The discrepancy between vasodilators and vasoconstrictors may have a vital role in the ED's pathogenesis. Therefore, endothelial dysfunction can be an important factor in ED in diabetic patients.

Penile erection is achieved by a hemodynamic process in which arterial inflow is increased, and venous outflow is restricted, resulting in a Penile Erection. ED may not be a life-threatening condition, but this communal problem can drastically change the quality of life and exert a negative impact on social and psychological social well-being.

In the pathogenesis of ED, a recent modification of multiple angiogenic and apoptotic factors has been implicated. Vascular endothelial growth factor (VEGF) has been widely studied in numerous complications of diabetes for its pathogenic importance, and we have already recorded that VEGF signaling in a rat model of type II diabetes is significantly reduced in the penis [8]. VEGF has demonstrated enhancement of the total endothelial and smooth muscle cell dysfunction in ED models [9]. Regular sildenafil administration in the penis of DMED rats can regenerate the compromised VEGF system and eventually enhance both erectile and endothelial function, indicating a possible general pathway for enhanced signaling via the VEGF / eNOS signaling cascade [10].

Endothelin function is also altered in all of the well-known complications of diabetes, including atherosclerotic and ischaemic disease, retinopathy, nephropathy, erectile dysfunction, and neuropathy. Increased endothelin levels (ET-1) and endothelin receptor upregulation (ETA and ETB) in the corpus cavernosum lead to penile vasoconstriction [11,12]. Such effects contribute to ultra-structural changes in lesions similar to atherosclerotic. The vasoconstriction induced by ET-1 is also linked to the RhoA / Rho-kinase pathway. This pathway mediates ED by restricting NOS and reducing NO generation in the corpora cavernosa [13-15]. Multiple action mechanisms are proposed, including transmembrane calcium flux, calcium sensitization via the Rho-Rho kinase pathway, and inositol triphosphate-sensitive intracellular calcium stores. The precise role of endothelins in ED's pathogenesis remains uncertain at present.

The present research used a three-week streptozotocin (STZ)-induced diabetic (DM) rat model to determine the expression of

VEGF with NO mechanism in penile tissue and subsequently investigated the influence of endothelin antagonism on these changes.

## Materials and Methods

### Animals and Drug treatment

Sprague-Dawley rats, Male, 10-week-old, were collected from Charles River Japan, Inc. (Yokohama, Japan) and cared for in compliance with the 1964 Helsinki Declaration Guiding Principles for the Caring and Use of Animals. The diabetic rats received a single intraperitoneal streptozotocin (STZ) injection of 65 mg/kg (Wako Pure Chemical Industries, Ltd., Osaka, Japan) dissolved in a 0.1 mol / L citrate solution (pH 4.5). In Non-diabetic animals (the control group), only the citrate buffer was administered. Animals having blood glucose levels higher than 250 mg / dL were classified as diabetics 48 hours after the STZ injection. One week after the STZ injection, the diabetic animals were randomly split into two groups; one group administered a particular ET A receptor blocker (TA0201) at a dosage of 1mg / day/rat for a minimum of 2 weeks using an osmotic mini pump (Model 2004, Durect Company, Cupertino, CA) (DM+TA0201), while the vehicle group was treated with physiological saline only (DM+vehicle). Before the start of the drug treatment, blood glucose was determined almost every day, but after the treatment started, the diabetic status was assessed every week. The rats were fed standard laboratory chow and allowed free access to water in an air-conditioned room with a 12-hour light-dark cycle until sacrificed. After 2 weeks of treatment, rats were sacrificed under anesthesia, and the penile tissue was removed. The present experimental design was approved by the Tsukuba University School of Medicine Animal Care and Use Committee.

### Quantitative real-time PCR

Total tissue (penile) RNA isolated with Isogen (Nippon Gene, Toyama, Japan) by an acid guanidinium thiocyanate-phenol-chloroform extraction. Briefly, the penile tissue was homogenized with a Polytron tissue homogenizer (PT10SK/35, Kinematica, Lucerne, Switzerland) in Isogen (100 mg tissue/1 ml Isogen); The precipitated RNA was extracted with chloroform, isopropanol precipitated, and ethanol washed with 75 percent (vol/vol). The resulting RNA was dissolved in diethyl pyrocarbonate-treated water, processed with DNase I (Takara, Shiga, Japan), and removed with Isogen again to isolate the genomic DNA again. The concentration of RNA was spectrophotometrically calculated at 260 nm. Total tissue RNA was primed with 0.05µg of oligo d (pT)12-18 and reverse transcribed using a first-strand cDNA synthesis kit (Qiagen) with omniscrypt reverse transcriptase. For 60 minutes, the reaction was conducted at 37 C.

The mRNA expression levels of VEGF and ET (A and B) receptors in the penile tissues were analyzed by quantitative RT-PCR with TaqMan probe using an ABI Prism 7700 Sequence Detector (Perkin-Elmer Applied Biosystems, Foster, CA, USA) as previously described (Maeda S). The gene-specific primers and TaqMan probes were synthesized from Primer Express

v.1.5 software (Perkin-Elmer) according to the published cDNA sequences for each of the following: Bcl-2, Bax, Caspase-3, and GAPDH mRNA. The sequences of the oligonucleotides were as follows:

VEGF

VEGF forward: 5'- TGAGACCCTGGTGGACATCTT -3'

VEGF reverse: 5'- CACACAGGACGGCTTGAAGA -3'

VEGF probe: 5'- CCCCATGAGATAGAGTAT -3'

ET-A

ET-A forward: 5'- GAATCTCTGCGCTCTCAGTGT -3'

ET-A reverse: 5'- GAGACAATTTCAATGGCGGTAATCA -3'

ET-A probe: 5'- CAGGAAGCCACTGCTCT -3'

ET-B

ET-B forward: 5'- GCTGGTGCCCTTCATACAGA -3'

ET-B reverse: 5'- CTTAGAGCACATAGACTCAACACTGT -3'

ET-B probe: 5'- ATCCCCACAGAAGCCT -3'

GAPDH

GAPDH forward: 5'-GTGCCAAAAGGGTCATCATCTC-3'

GAPDH reverse: 5'-GGTTCACACCCATCACAAACATG-3'

GAPDH probe: 5'-TTCCGCTGATGCCCC-3'

GAPDH mRNA expression was used in the present study as an internal control. The PCR mixture (25 µl volume) consists of 450 nM for both GAPDH (Perkin-Elmer) forward and reverse primers, 200 nM for FAM-labeled primers (Perkin-Elmer), and TaqMan Universal PCR Master Mix (Perkin-Elmer). Every PCR amplification was conducted in triplicate using the following profile: 1 cycle of 95°C 10 min, and 40 cycles of 94°C for 15 sec and 60°C for 60 sec. For the standard real-time quantitative PCR curve, sequential dilutions of a rat penis cDNA were performed at different concentrations (1X, 2X, 4X, 8X, and 16X). No template mixture (water) prepared as a negative control reaction.

### Enzyme Immunoassay for penile ET-1

An ET-1 Enzyme Immuno Assay Kit (Immuno-Biological Laboratories, Fujioka, Japan) was used to assess the concentration of ET-1 in penile tissue extracts. This kit is a solid-phase ELISA sandwich that uses 2 forms of highly selective antibodies. Tetraethyl benzidine (TMB) is used as a coloring agent (Chromogen), whose power is proportional to ET-1 amounts. An equivalent volume of 0.1 percent trifluoroacetic acid (TFA) in water was applied to the frontocortical tissue sample. The sample was then centrifuged at 3,000 g to clear at 4°C for 15 minutes and saved the supernatant. After a 200 mg, C 18 Sep-Pak column (Waters Inc. Part No. 235D1) was washed in 0.1 percent TFA with 4x1 ml of 60 percent acetonitrile followed by 4x5 ml of 0.1 percent TFA in water, the supernatant was added to the column and washed in water with 4x5 ml of 0.1 percent TFA. The sample was slowly eluted by applying 3x1 ml of 60 percent acetonitrile in 0.1 percent TFA in water and collecting the eluant in a plastic tube. The eluant was then evaporated under vacuum using a centrifugal concentrator to dryness. The sample was reconstituted with assay buffer and subsequently weighed according to instructions from the manufacture.

### Enzyme Immunoassay eNOS

Penile tissues from the sample groups were homogenized, centrifuged, and after centrifugation, the concentration of proteins was measured in the supernatant. These procedures were carried out in conjunction with the instructions of the Immunoassay kits used in the tissue extracts for eNOS protein detection. For the determination eNOS, the kits were purchased from R&D system. (Minneapolis, MN 55413, US) The Immunoassay Enzyme was performed according to instructions from the manufacturer.

### Nitric oxide colorimetric assay

Indirectly, nitric oxide (NO) was observed as nitrite in penile tissue extracts using a NO Colorimetric Assay kit (Roche Diagnostics, Mannheim, Germany). In this process, in the presence of the enzyme nitrate reductase, the nitrate present in the sample was reduced to nitrite by decreasing the nicotinamide adenine dinucleotide phosphate. Formed with sulfanilamide and N-(1-naphthyl) ethylenediamine dihydrochloride, the nitrite reacted to give a red-violet diazo dye. Due to its absorbance within the visible range, the diazo dye was measured at 550 nm.

### Statistical analysis

Values are means ± SD. Statistical assessment of the data was made by one-way analysis of variance with multiple comparisons by Fisher's protected least-significant difference t-test. Non-parametric data in the current study were analyzed by the Mann-Whitney U test or Wilcoxon signed-rank test. P<0.05 was taken as significant.

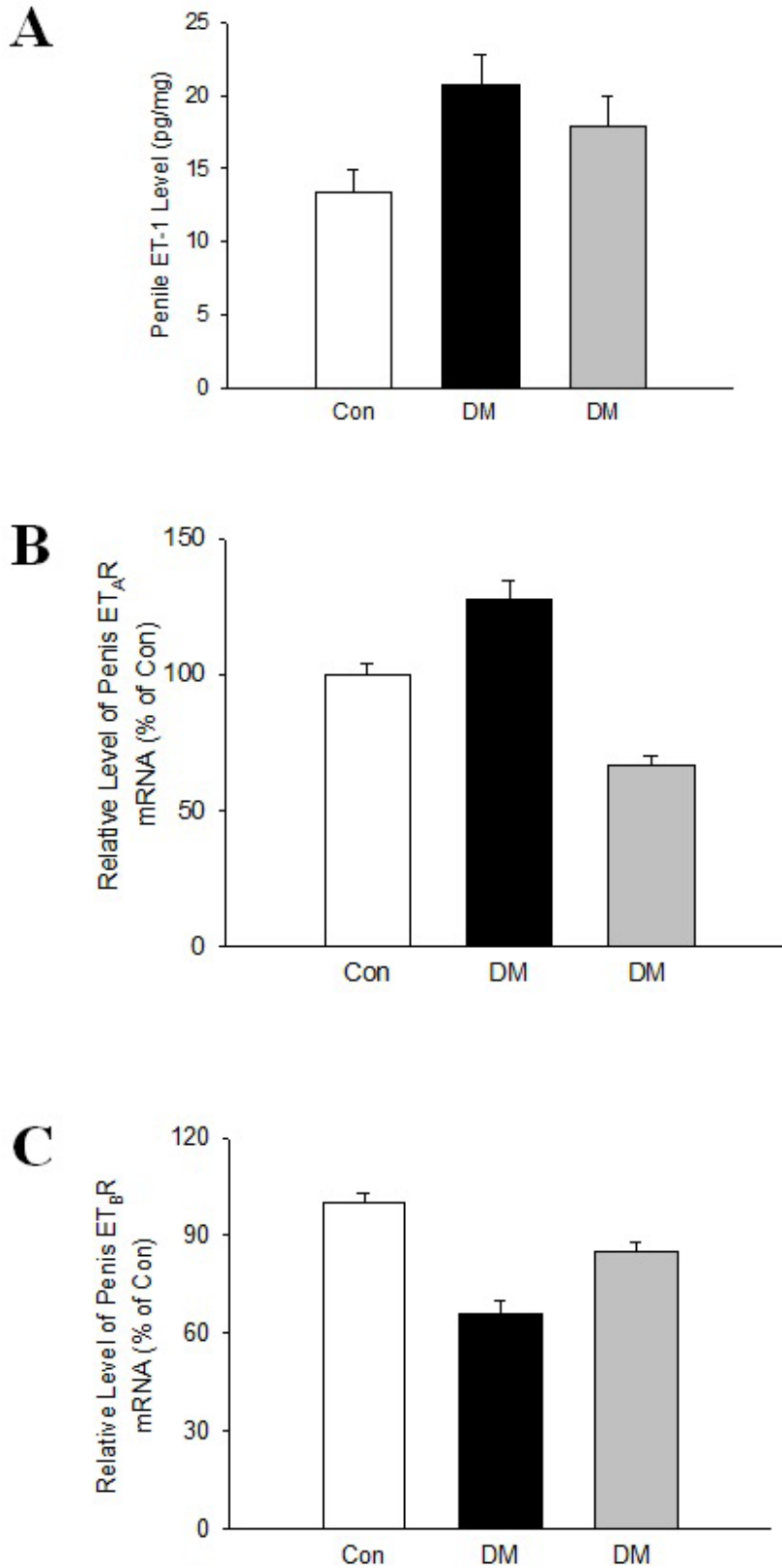
## Results

### Biochemical and physical parameters of the experimental animals

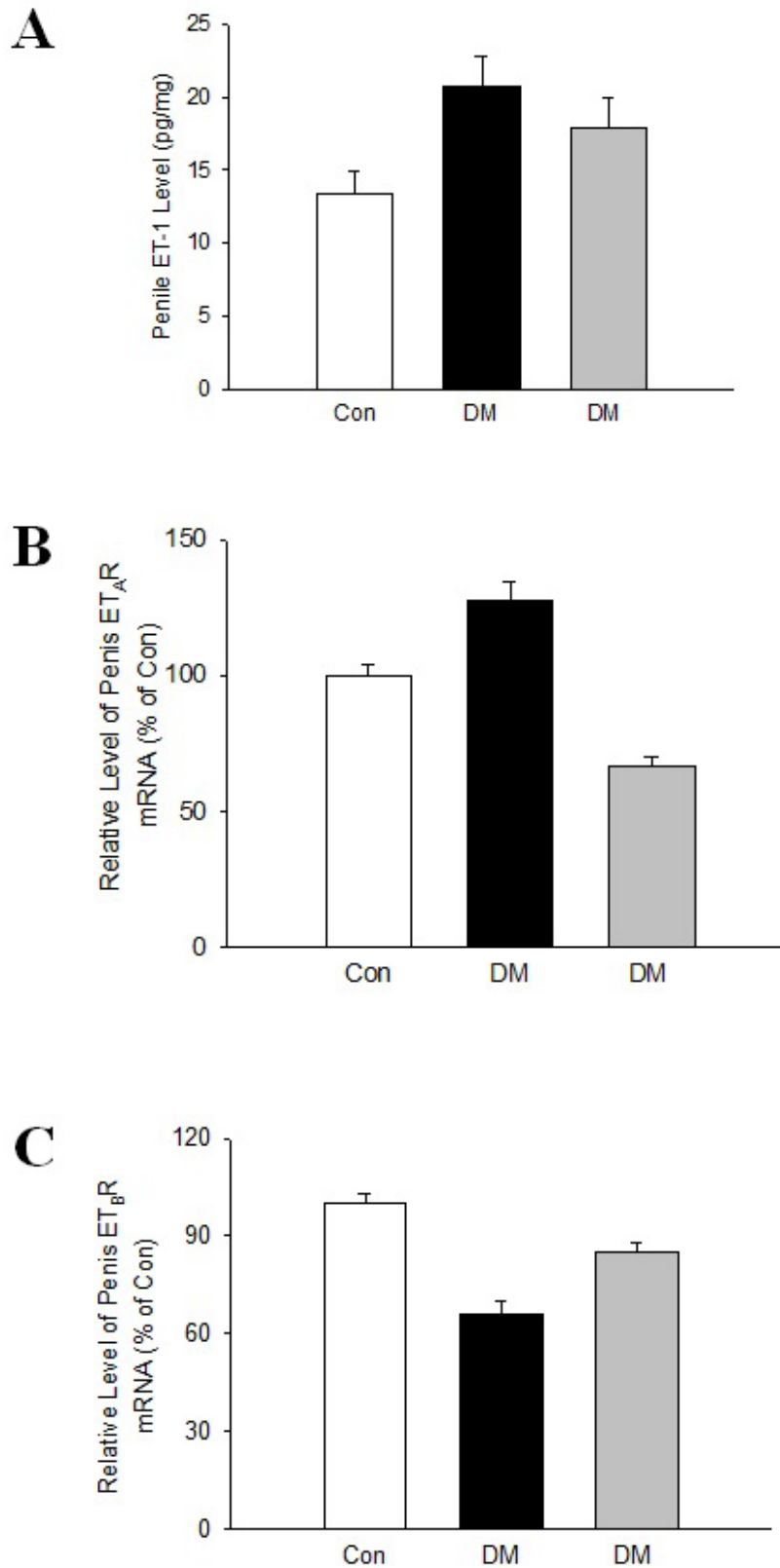
Kidney to body weight and left ventricular to body weight ratios were significantly increased in DM rats and were partly reversed by ET antagonist. Glucose levels in DM rats significantly increased (472 ± 79 mg/dL) than in non-DM rats (116 ± 7 mg/dL). The plasma insulin level was remarkably decreased in DM rats (0.32 ± 0.30 ng/ml) compared to non-DM control animals (7.94 ± 2.44 ng/ml). Treatment of DM rats with ET antagonist for 2 weeks altered neither hyperglycemia nor hypoinsulinemia Systolic and diastolic blood pressure was not changed among the experimental animals. There was no urinary excretion of protein. But there was profuse urinary excretion of glucose, and this was not influenced by ET antagonism. At this stage of diabetes, there was no abnormality of heart in echocardiography.

### Effects of ET antagonist on VEGF signaling system

A 30% decrease in VEGF expression in penile tissue was seen in DM rats (Figure 1A), ET antagonist significantly recovered this downregulation. Both of the phosphorylated NO and eNOS has been well documented as downstream molecules of VEGF angiogenic signaling cascade. Plasma NO level was unchanged in current investigation (data not are shown), but penile NO and eNOS level was decreased in DM rats, whereas greatly improved by ET-A receptor antagonist (Figures 1B and 1C).



**Figure 1 (A):** The mRNA expression of VEGF in the penile tissues of control, DM+vehicle, and DM+TA-0201 rats. The mRNA expression was determined by real-time PCR. Data are means  $\pm$  SD (n=10). The data are shown as the relative levels (the value of the Control is defined as 100%). \*P<0.01 vs. Control; #P<0.01 vs. DM+vehicle. **(B, C):** NO, and eNOS ELIZA in the penile tissues of control, DM+vehicle, and DM+ TA-0201 rats. Data are shown as means  $\pm$  S.D. Statistical analysis was conducted using one-way ANOVA followed by Fisher's protected least significance t-test. \*P<0.01 vs.Control; #P<0.01 vs. DM+vehicle.



**Figure 2 (A):** ET-1 ELIZA in the penile tissues of control, DM+vehicle, and DM+ TA-0201 rats. Data are shown as means  $\pm$  S.D. Statistical analysis was performed using one-way ANOVA followed by Fisher's protected least significance t-test. \* $P < 0.01$  vs. Control; # $P < 0.01$  vs. DM+vehicle. **(B, C):** The mRNA expression of ET-A and ET-B receptors in the penile tissues of control, DM+vehicle, and DM+TA-0201 rats. The mRNA expression was determined by real-time PCR. Data are means  $\pm$  SD (n=10). The data are shown as the relative levels (the value of the Control is defined as 100%). \* $P < 0.01$  vs. Control; # $P < 0.01$  vs. DM+vehicle.

## Expression of ET system

Penile ET-1 level was increased in the DM group and was decreased by ET antagonist (Figure 2A) significantly. ETAR in penile tissue in early diabetes was increased by 27% and was remarkably downregulated by ET antagonism (Figure 2B). ETBR expression was downregulated in the early DM penis and was recovered by ET antagonist (Figure 2C).

## Discussion

The present study demonstrated that STZ-induced diabetic rats had decreases in VEGF and its signaling molecules (eNOS and NO) expression in penile tissues at 2 to 3 weeks of diabetes. This change was associated with the penile upregulation of ET-1 in DM rats. At this diabetic stage, the DM rats exhibited remarkable insulin reduction in plasma as well as high plasma glucose levels. And ET antagonism by a selective ET-A receptor could significantly reverse the downregulated VEGF system in penile tissues in DM rats.

ED aetiopathogenesis in diabetes is multifactorial, with a significant presence of vascular and neuronal causes. We also recently stated that the expression of the vascular endothelial growth factor (VEGF) in diabetic type II penile tissues [11] is downregulated. There is strong evidence that endothelial cells have VEGF as a survival factor [16]. This pro-survival activity of VEGF involves the PI3K / Akt signal transduction pathway, and activation of Akt induces the expression of Bcl-2, an anti-apoptotic protein, a pro-apoptotic protein, phosphorylation of Bad, thus inhibiting execution of apoptosis.

As expected from finding the reduced expressions of Bcl-2, DM rats in this study showed reduced expressions of VEGF, its receptors, and Akt in penile tissues (Unpublished Observation). The antiapoptotic protein Bcl-2 plays a significant role in inhibiting extrinsic and intrinsic mitochondria-dependent cell death pathways [17]. The present findings are in good agreement with the previous study, which showed the loss of Bcl-2 expression from diabetic men with ED in cavernosal tissue [18].

Compared to those in the control group, apoptotic cells were more abundant ( $P < 0.01$ ), and Bcl-2 expression was absent in the diabetic rat's penis cavernosal tissues [19]. Our findings also support the increased number of apoptotic cells in the erectile tissues of diabetic rats induced by STZ. Although extensive research is required to determine the function of apoptosis in the pathophysiology of diabetic ED, we think that apoptosis may cause some loss of erectile tissue within the corpora, contributing to the degeneration of penile cavernosum by replacing the cavernosal smooth muscle with collagen.

Increased development of the strong vasoconstrictive, mitogenic, and pro-inflammatory peptide endothelin-1 (ET-1) was consistent with endothelial dysfunction in diabetes and insulin-resistant diabetic conditions [17]. ET-1 is synthesized in the human penile corpus cavernosum by smooth muscle and endothelial cells [5,10] and the peptide will cause both vasoconstriction and vasodilatation

in the penis, with ETA receptors mediating ET-induced cavernous vasoconstriction, while ETB receptors mediate vasodilatation by releasing NO from cavernous endothelial cells. ET-1 levels in plasma and cavernous sinusoids are elevated in diabetic people with ED, and up-regulation of both ETA and ETB receptors in cavernosal tissue from type 1 diabetic rats and rabbits and insulin-resistant obese Zucker rats (OZR) has been shown.

In the pathogenesis of ED, the interaction between vasoconstrictors and vasodilators may play a significant role. Plasma levels of ET-1, ACE activity are elevated and associated with a reduction of NO and cGMP levels in ED patients' systemic and cavernous blood [20]. In this study, the level of plasma ET-1 was not increased, but the level of penile ET-1 was increased, and this upregulation was normalized by antagonism to the ET. The adrenergic agonist norepinephrine (NE) [21] and ET-1 [22] are two vasoconstrictors that are believed to play an important role in controlling the blood flow of the penile. Thus decreasing the amount of penile ET-1 can partially increase the blood flow in penile tissues. And improving blood flow in several angiogenic and apoptotic factors involved in ED pathogenesis could normalize the changes. The mechanism of recovery of downregulated Bcl-2 expression in DM penis by antagonism to ET is hard to determine as several experiments have shown that ET-1 inhibits apoptosis. ET-1 does not reverse the drop in levels Bcl-2 in irradiated human melanocytes.

In conclusion, the present study explores that the expression of an anti-apoptotic molecule, Bcl-2, is substantially reduced in early diabetes penile tissues and dual ET antagonism may reverse this downregulation, whereas the present study cannot shed light on the mechanism leading to the reversal of Bcl-2 downregulation in the diabetic penis through antagonism with ET.

## Conclusion

From the above observations, it can be mentioned that antagonism with ET may be helpful to normalize the component parts of ET and NO systems, the essential vasoregulatory systems for ED, in the early DM penile tissues, and also effective in the normalization of VEGF loss in early DM penile tissues.

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