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ORIGINAL ARTICLE

Therapeutic effect of combination of alagebrium (ALT-711) and sildenafil on erectile function in diabetic rats

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Recently, the relationship between advanced glycation end products (AGEs) and erectile dysfunction (ED) has been reported. The present study aimed to investigate whether a combination of an AGE cross-link breaker (alagebrium/ALT-711) and sildenafil could enhance the erectile capacity in streptozotocin (STZ) diabetic rats. Additionally, we assessed the effect of that treatment option on some molecules that have been suggested to have crucial roles in AGE-related ED pathways. Four groups of animals were utilized: (1) age-matched control rats, (2) STZ-induced diabetic rats (40 mg kg^{-1} i.p.), (3) STZ rats + sildenafil (5 mg kg^{-1} p.o.), (4) STZ rats treated with a combination of sildenafil (5 mg kg^{-1} p.o) + alagebrium/ALT-711 (10 mg kg^{-1} p.o.) for the final 1 month of the 2 months of diabetes period. At 2 months after i.p. injection of STZ, all animals underwent cavernosal nerve stimulation (CNS) to assess erectile function. Penile tissue AGEs, MDA (malondialdehyde), cyclic guanosine monophosphate (cGMP) (ELISA), endothelial nitric oxide (NO) synthase (eNOS), inducible NO synthase (iNOS) (western blot), nuclear factor (NF)-κB, mitogen-activated protein (MAP) kinase (immunohistochemistry) and apoptosis (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling) analyses were performed in all groups of rats. STZ diabetic rats had a significant decrease in erectile function as determined by the peak intracavernosal pressure (ICP) and total ICP (area under the erectile curve) after CNS when compared with control rats ($P<0.05$). The increase in both ICP and area under the erectile curve of STZ diabetic rats treated with a combination of sildenafil + alagebrium/ALT-711 as well as in STZ diabetic rats treated with sildenafil alone was significantly greater than STZ diabetic rats. Additionally, combination treatment decreased AGE, MDA, iNOS, NF-κB, MAP kinase and apoptosis levels, whereas it preserved cGMP contents in diabetic penile tissue. Decreased AGE, MDA, iNOS, NF-κB, MAP kinase and increased cGMP levels at the combination (sildenafil + alagebrium/ALT-711) therapy group increased both the peak ICP and total ICP to CNS in the STZ diabetic rats, which was similar to the response observed in control rats. These results may explain the role of AGEs in diabetes-related ED and the effect of an AGE cross-link breaker alagebrium/ALT-711 + sildenafil therapy on some critical molecules related to AGE-related ED pathways.

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INTRODUCTION

Erectile dysfunction (ED), the consistent inability to attain or maintain an erection for satisfactory sexual intercourse is more commonly experienced in diabetic men than the general population across all ages.[1] Recently, it has been reported that >6% of the USA population has diabetes, of this, approximately 8 million people have some degree of ED.[2] Although, the exact mechanism of ED in diabetic men is not fully understood, autonomic neuropathy, endothelial dysfunction and hormonal imbalance are believed to be primarily responsible for ED rates, reported as high as 50–75%.[1] Penile erection is a complex dynamic event that involves the relaxation of the smooth muscle cells of the corpus cavernosum and related arteriols. In several studies it has been shown that neuronal and endothelial nitric oxide (NO) is the principal mediator of cavernosal smooth muscle relaxation and penile erection. Furthermore, substantial evidence supports the existence of impairment in both neurogenic and endothelium-dependent cavernosal smooth muscle relaxation in diabetes mellitus (DM).[3] Recent investigations in which molecular biological techniques have been used, provided researchers with new insights into the pathophysiological mechanism of diabetes-related ED. Elevated levels of advanced glycation end products (AGEs), increased levels of reactive oxygen species, impaired NO production, decreased and impaired cyclic guanosine monophosphate (cGMP)-dependent kinase-1, increased endothelin B receptor-binding sites, upregulated RhoA/Rho-kinase pathway and NO-dependent selective nitrergic nerve degeneration are some of the proposed mechanisms of diabetes-related ED.[2]

AGEs develop in diabetic penile tissue secondary to hyperglycemia. AGE formation is the result of nonenzymatic glycation between reduced sugars such as aldoses and ketoses, and free amino groups of proteins, lipids and nucleic acids. The ultimate result of this reaction leads to pathological cross-linking, which causes to impair in tissue remodeling. The most important physiological consequences of these pathological processes are vascular thickening, decreased elasticity, endothelial dysfunction and atherosclerosis.[4] Furthermore, it has been reported that AGEs quench both endothelial and neuronal-derived NO decreasing cavernosal smooth muscle relaxation. Increased AGEs in diabetic penile tissue, blocks the interaction between NO and guanylate cyclase, which results in decreased cGMP levels and ultimately

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Table 1. The mean of body weight and BG concentrations for each group before and 2 months after injection of STZ

	Control	*Diabetic*	*Diabetic+sildenafil*	*Diabetic+sildenafil+ALT*
Mean wt ± s.e.m. (gm):				
Initial	355 ± 12	360 ± 9	345 ± 11	340 ± 15
Final	465 ± 15[a]	320 ± 14	305 ± 13	310 ± 18
Mean of BG ± s.e.m. (mg dl^{-1})				
Initial	102 ± 4.7	155 ± 5.2	100 ± 5.4	110 ± 6.1
Final	104 ± 5.6	492 ± 9.1[a,b]	517 ± 10.8[a,b]	524 ± 11.4[a,b]

Abbreviations: BG, blood glucose; STZ, streptozotocin.
Seven animals per group.
[a]$P<0.05$ significantly different from initial value in the same group.
[b]$P<0.05$ significantly different from final value in the control group.

impaired smooth muscle relaxation. Several studies showed a significant increase in glycation, AGEs, galectin-3 (specific AGE receptor) and inducible NO synthase (iNOS) protein levels in streptozotocin (STZ) diabetic rat penises.[4,5] Furthermore, previously it has been shown that both inhibitors and cross-link breakers of AGEs have protective and therapeutic effect on erectile function in diabetes-induced rats.[4,6]

Sildenafil is an orally active selective type 5 phosphodiesterase (PDE) inhibitor, and has proven to be an effective agent for the treatment of male ED. Sildenafil inhibits the breakdown of intracellular cGMP and facilitates NO-mediated corpus cavernosum smooth muscle relaxation, which leads to enhanced erectile responses. Although the efficacy of sildenafil in general population has been reported as 74–97%, it is only 50–56% among diabetic individuals.[7,8]

As mentioned before, the effect of both AGE inhibitors and cross-link breakers has already been shown in various experimental studies.[4-6] On the other hand, the potential impact of combining AGE cross-link breaker namely ALT-711 and sildenafil to treat diabetes-related ED remains to be determined. In the present experimental study, we aimed to gain insights into the potential clinical utility of this combination treatment. Additionally, we assess the effect of that treatment modality on penile tissue AGE (5-hydroxymethyl-2-furaldehyde) and reactive oxygen species such as malondialdehyde (MDA). Furthermore, we evaluated the impact of the combination therapy on some molecules including iNOS, endothelial NO synthase (eNOS), c-GMP, nuclear factor(NF)-κB and mitogen-activated protein (MAP) kinase, which have been suggested to have crucial roles in AGE-related ED pathways. Finally, we investigated whether apoptosis of corpora cavernosa smooth muscle cells and erectile function are recovered by combination of ALT-711 and sildenafil in diabetic rats.

MATERIALS AND METHODS

Study design

The study was mainly done in 28 adults, 18–20-weeks old male Wistar rats. The Akdeniz University Animal Ethics Committee approved the research protocol. The animals were randomly divided into four groups: (1) age-matched control group ($n=7$) receiving citrate buffer, pH 7.0, by i.p. injection, (2) diabetic group ($n=7$) receiving i.p. injection of 40 mg kg^{-1} dose of STZ (Sigma Chemical Company, St Louis, MO, USA) in citrate buffer, and treatment groups ($n=7$) receiving 5 mg kg^{-1} per day (p.o) of sildenafil citrate (Pfizer, New York, NY, USA) either alone (3) or combined with ALT-711 (Alteon, Parsippany, NJ, USA) 10 mg kg^{-1} per day (p.o), (4) for the final 1 month of 2 months of diabetes period. The total body weight and blood glucose levels were determined before and after i.p. injection of STZ with Accu-check blood glucose meter (Roche Diagnostics, Indianapolis, IN, USA). Animals having blood glucose levels >200 mg dl^{-1} were considered diabetic. The blood glucose levels and total body weights are summarized in Table 1. While 5-hydroxymethyl-2-furaldehyde (5-HMF), MDA and cGMP levels were measure in four animals from each group (penises were divided in three pieces except the glans penis), western blot analyses were performed in the remaining three animals from each group.

Additionally, for the immunohistochemical (IHC) analyses we used 24 additional animals, which were divided as the mentioned four groups (NF-κB, MAP kinase and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay; control, DM, DM + sildenafil and DM + sildenafil + ALT-711 groups).

Measurement of cavernosal AGE (5-HMF) levels

The AGE level was determined from the 5-HMF content in penis samples of control, STZ diabetic and both therapeutic groups, sildenafil alone and combined with ALT-711-treated tissue of STZ diabetic rats ($n=4$ samples from each group), by the colorimetric method of Kasai *et al*.[9] Briefly, 20 mg penile tissue was hydrolyzed in 0.33 M oxalic acid by boiling for 5 h and then ice-cold 40% trichloroacetic acid was added to precipitate protein. The supernatant obtained after centrifugation at $1500\times g$ for 10 min was combined with 0.05 M thiobarbituric acid and incubated at 37 °C for 30 min. The absorbance ($\lambda=443$ nm) was measured at the end of incubation time. 5-HMF levels were expressed as nmol mg^{-1} tissue.

Measurement of cavernosal MDA levels

MDA, a product of lipid peroxidation, level was determined in samples of all four groups ($n=4$ samples from each group), by the fluorometric method of Wasowicz *et al*.[10] Briefly, 20 mg tissue homogenized in 0.15 M sodium phosphate buffer, pH 7.4, was reacted with thiobarbituric acid by boiling for 1 h. Followed by adding butanol, the fluorescence of supernatant obtained after centrifugation at $2500\times g$ for 10 min was measured at $\lambda_{ex}=525$ nm and $\lambda_{em}=547$ nm. MDA levels were expressed as nmol mg^{-1} protein. Protein amounts were quantified by Lowry methods based on a complex formation of divalent copper ion with peptide bonds in which it is reduced to a monovalent ion under alkaline conditions.[11]

Detection of cavernosal GMP levels

Cavernosal cGMP levels were measured in control and STZ diabetic corpora, and both therapeutic groups ($n=4$ samples from each group). For the determination of cGMP levels, cavernosal samples were assayed by using an enzyme-linked immunoassay kit (Cayman Chemical Company, Ann Arbor, MI, USA) based on the competition between free-cGMP and a cGMP-acetylcholinesterase conjugate for a limited amount of cGMP-specific rabbit antibody-binding sites, as previously described.[12]

Immunoblot analysis of iNOS and eNOS in cavernosal tissue

Cavernosal tissues from control, STZ diabetic rats, and both therapeutic groups that are sildenafil alone and combine with ALT-711-treated STZ diabetic rats ($n=3$ samples from each group) were homogenized with a Brinkmann Polytron (Brinkmann Instruments, Westbury, NY, USA) in lyses buffer with 1 mM phenylmethanesulfonylfluoride and sonicated for 30 s followed by a incubation period of 30 min on ice. iNOS protein expression

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analysis was performed in cytosolic fraction of each sample. The membrane fraction isolated by 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) was used for eNOS protein expression because it contains ~80% eNOS.[13] Equal amounts of protein, 40 μg, were run on 7.5% SDS/polyacrylamide gels and transferred electrophoretically to 0.2 μm nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). Nonspecific binding sites were blocked with 5% milk in Tris-buffered saline Tween-20 (TBST) (120 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.05% Tween 20) for 1 h at room temperature. The membranes were first incubated with polyclonal rabbit anti-iNOS (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or monoclonal rabbit anti-eNOS primary antibody (1:1000 dilution; Cell Signaling Technology, Beverly, MA, USA) overnight at 4 °C, and then with horseradish peroxidase-conjugated secondary antibody (1:1000 dilution; Biosource, Carlsbad, CA, USA) for 1 h at room temperature. The bands were visualized by using enhanced chemiluminescence detection reagent kit (Amersham Biosciences, Arlington, IL, USA). Equal loading and transfer were confirmed by blotting the same membrane with monoclonal mouse anti-GAPDH primary antibody (1:5000 dilution; Chemicon International, Temecula, CA, USA). Quantification of band intensity was performed by Image J version 1.24 software (developed at the National Institutes of Health, Bethesda, MD, USA). Data are representative of five independent experiments with nearly identical results.

IHC localization of NFκB-p65 and MAP kinase-p38

For NFκB-p65 and MAP kinase-p38 immunohistochemistry, cavernosal tissues were removed from control rats, STZ diabetic rats, STZ diabetic rats treated with sildenafil alone and combination of sildenafil + ALT-711 rats ($n = 2$ samples from each group). For IHC analyses, tissues were fixed in freshly prepared 4% paraformaldehyde and embedded in paraffin. A 4-μm cross-sections were deparaffinized in xylene and rehydrated by baths of graded series of alcohol, distilled water and phosphate-buffered saline (PBS). They were subjected to antigen retrieval by boiling the sections in 10 mM citrate buffer, pH 6.0, at 125 °C microwave for 15 min and then cooled at room temperature. After rinsing in PBS, the sections were incubated in 3% H_2O_2 in methanol for blocking endogenous peroxidase activity. The sections were first incubated in ultraviolet-blocking solution for 5 min at room temperature to prevent nonspecific binding, and then with monoclonal mouse anti-NFκB-p65 (1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or polyclonal rabbit anti-MAP kinase-p38 primary antibody (1:200 dilution; Santa Cruz Biotechnology) or rabbit-negative control overnight at 4 °C. Detections were done by using appropriate biotinylated secondary antibodies for 30 min and avidin-biotin complex for 15 min at room temperature (ABC; Vector Laboratories, Burlingame, CA, USA), with 3′3′-diaminobenzidine (DAB, Sigma-Aldrich, St Louis, MO, USA) as chromogen for 10 min. The sections were counterstained with Mayer's hematoxylin and mounted using mounting medium. Negative-control slides using lymph node tissue were included with each antibody. All slides were examined by light microscopy, and the presence of labelled cells was documented and tabulated. The absence of staining was documented as a negative result (−) and the presence of staining as a positive result (+) using a scale based on the number of cells per high-power field (× 200), as previously reported: +, 1-4; ++, 5-10; +++, >10; ++++ and >100.[14]

Apoptosis using TUNEL assay

The rates of cavernosal cells apoptosis were evaluated using ApopTag Plus Peroxidase *in situ* Apoptosis Detection Kit (Chemicon International Temecula, CA, USA) on the basis of the TUNEL method, according to the manufacturer's specifications. Briefly, the deparaffinized cavernosal tissue ($n = 2$ samples from each group) sections (4 μm thick) were incubated with proteinase K (20 μg ml^{-1}) for 15 min at room temperature and washed with PBS. The sections were equilibrated with terminal deoxynucleotidyl transferase, recombinant (rTdT) reaction mixture (25 mM Trizma base pH 6.6, 200 mM potassium cacodylate pH 6.6, 0.2 mM dithiothreitol, 0.25 mg ml^{-1} bovine serum albumin and 2.5 mM cobaltchloride) for 10 min. Then they were covered with the biotinylated nucleotide in presence of rTdT enzyme and incubated in a humidified chamber at 37 °C for 60 min. The reaction was terminated by transferring the slides into 2 × saline sodium citrate buffer, pH 7.0, for 15 min at room temperature. Endogenous peroxidase was inactivated by incubating with 3% H_2O_2 for 5 min. The sections were rinsed with PBS and incubated with streptavidin-horseredish peroxidase (1:500 dilution) for 30 min. After washing in PBS, the sections were incubated with diaminobenzidine substrate solution with peroxidase and counterstained with Harris's hematoxylin. IHC expression of TUNEL-positive cells shows in the nuclei. The mean percentage of positive cells was determined from five areas at high-power field (× 400). The apoptosis index were calculated by counting the TUNEL-positive cells in a total of 1000 cells observed from more than representative high-power fields, respectively. The percentage of positive cells was calculated as the ratio of positive cells to the total number of cells. The scoring scale for the percentage of positive cells was: 0, <1%; 1, 1-24%; 2, 25-50%; 3, 51-75% and 4, >75%. The scoring scale for staining intensity was: 0, no color; 1, bright yellow; 2, yellow; 3, brown yellow and 4, brown. The final score was obtained by multiplying the percentage of positive cells by the staining intensity score.

Measurement of erectile responses

At 2 months after diabetes induction, intracavernous pressure measurements were determined in the four experimental groups of animals, as previously described in several studies.[3-6]

Statistical analysis

The data are expressed as mean ± s.e.m. Statistical analyses were performed using a software program for statistics (SPSS 13.0 Version, SPSS Inc, Chicago, IL, USA). The differences between the groups were compared by using two-tailed Mann-Whitney *U*-test or one-way analysis of variance with repeated measures followed by Kruskal-Wallis test for multiple group comparisons. Level of significance <0.05 was considered significant.

RESULTS

Cavernosal AGE (5-HMF) and MDA levels

5-HMF served as a marker of penile AGE content, whereas MDA was considered as a marker for reactive oxygen species. As shown in Figure 1, both 5-HMF and MDA levels were significantly increased in the penile tissue of STZ diabetic rats and STZ diabetic rats treated with sildenafil alone. However, combination treatment (sildenafil + ALT-711) caused a significant decrease in 5-HMF and MDA levels in the penile tissue of STZ diabetic rats.

Cavernosal c-GMP levels

Cavernosal tissue concentrations of cGMP were measured in control, STZ diabetic rats and STZ diabetic rats treated with sildenafil and sildenafil + ALT-711, and these data are shown in Figure 2. Cavernosal cGMP levels were significantly lower ($P<0.05$) in the STZ diabetic rats and STZ diabetic rats treated with sildenafil alone when compared with control rats. Sildenafil + ALT-711 treatment significantly increased penile tissue c-GMP levels when compared with STZ diabetic rats and STZ diabetic rats treated with sildenafil alone ($P<0.05$).

Western blot analysis of iNOS and eNOS

Levels of iNOS, eNOS and GADPH protein expression were measured in rat cavernous tissue from control, STZ diabetic, STZ diabetic treated with sildenafil and STZ diabetic rats treated with sildenafil and ALT-711 (Figure 3). Penile tissue iNOS (130kDa) protein levels were significantly increased in cavernous tissue of STZ diabetic rats compared with control and STZ diabetic rats treated with either sildenafil alone or sildenafil + ALT-711 ($P<0.05$).

Furthermore, whereas penile tissue eNOS (140 kDa) protein levels were significantly decreased in cavernous tissue in STZ diabetic rats compared with control group ($P<0.05$), eNOS levels

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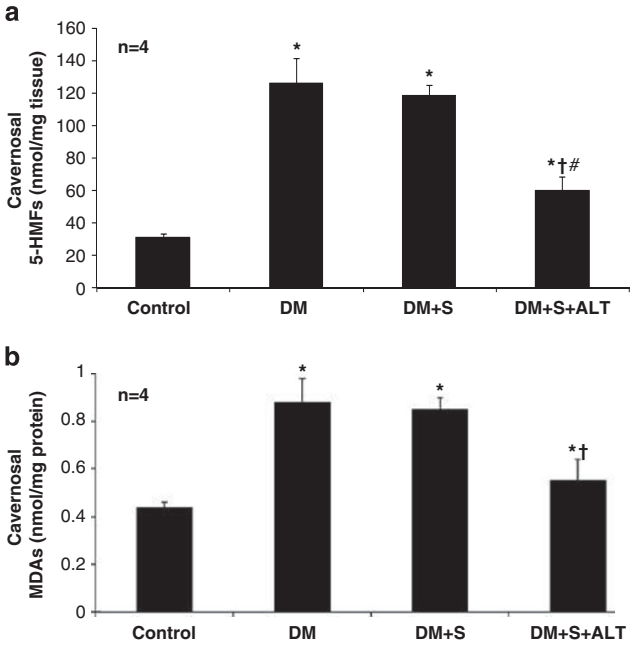


Figure 1. 5-HMF (**a**) and MDA (**b**) in cavernous tissue from control, STZ-induced diabetic rats, STZ-induced diabetic rats treated with sildenafil, and STZ-induced diabetic rats treated with sildenafil and ALT-711. * indicates $P<0.05$ versus control, † indicates $P<0.05$ versus DM and # indicates $P<0.05$ versus DM + S group. DM, diabetes mellitus; 5-HMF, 5-hydroxymethyl-2-furaldehyde; MDA, malondialdehyde; S, sildenafil; STZ, streptozotocin.

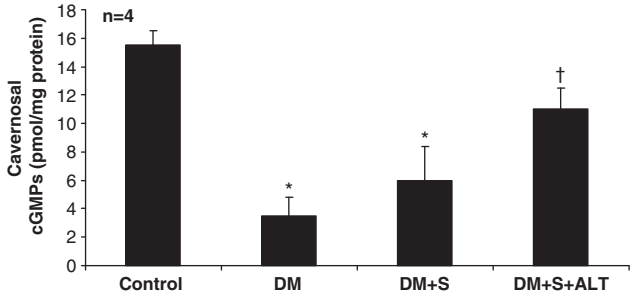


Figure 2. Bar graph demonstrating cGMP levels in cavernosal tissue of control, STZ diabetic rats, STZ diabetic rats treated with sildenafil alone and STZ diabetic rats treated with sildenafil + ALT-711. *$P<0.05$ indicates that cGMP levels are significantly different when compared with control rats. † $P<0.05$ indicates that cGMP levels are significantly different when compared with STZ-diabetic rats and STZ-diabetic rats treated with sildenafil. cGMP, cyclic guanosine monophosphate; DM, diabetes mellitus; S, sildenafil; STZ, streptozotocin.

were preserved in penile tissues from STZ diabetic rats treated with sildenafil alone. On the other hand, whereas penile tissue eNOS content in combination treatment group were greater than the STZ diabetic group, the difference between the two groups was not statistically significant ($P>0.05$).

IHC localization of NFκB-p65 and MAP kinase-p38

To determine the cellular localization of NFκB-p65 and MAP kinase-p38 in our four study groups, IHC localization was performed in control and STZ diabetic penises (group 2, 3 and 4) (Figure 4). IHC staining for NFκB-p65 and MAP kinase were

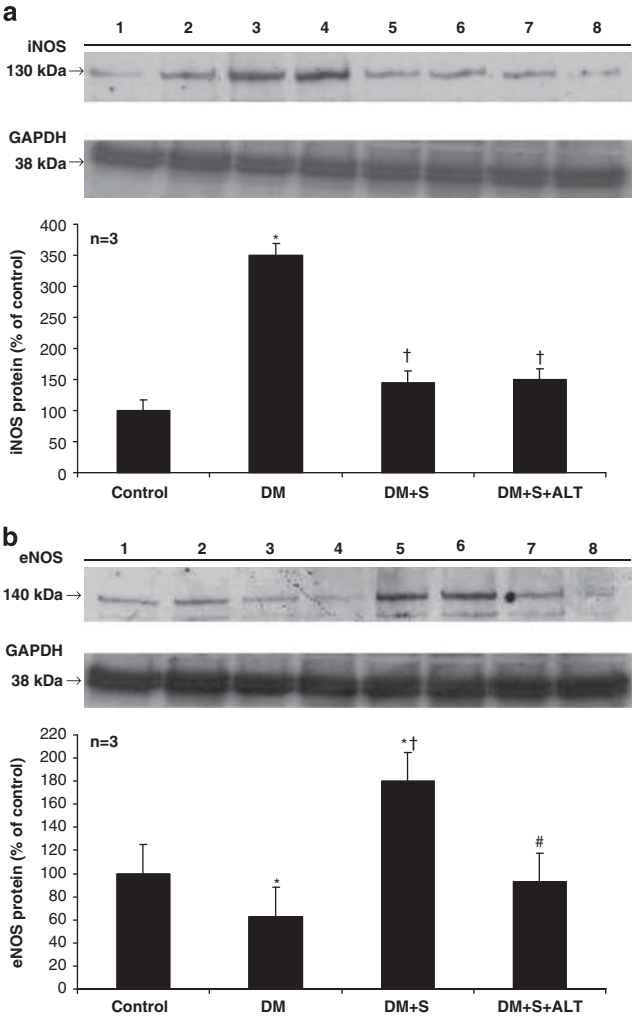


Figure 3. Western blot analysis (top) demonstrates expression of iNOS (**a**) at 130 kDa and eNOS (**b**) at 140 kDa, and in penile tissue of controls (lane1, 2), STZ diabetic rats (lane 3, 4), STZ diabetic rats treated with sildenafil (lane 5, 6) and STZ diabetic rats treated with sildenafil and ALT-711 (lane 7, 8). Densitometry analysis (bottom) of iNOS (**a**) (iNOS-to-GAPDH) and eNOS (**b**) (eNOS-to-GAPDH) protein in cavernous tissue of five controls, STZ diabetic rats, STZ diabetic rats treated with sildenafil and STZ diabetic rats treated with sildenafil + ALT-711. * indicates $P<0.05$ versus control, † indicates $P<0.05$ versus DM and # indicates $P<0.05$ versus DM + S group. DM, diabetes mellitus; eNOS, endothelial nitric oxide synthase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; iNOS, inducible nitric oxide synthase; S, sildenafil; STZ, streptozotocin.

predominantly localized to the cavernous sinusoids and vascular endothelium in control and STZ induced diabetic rats, and STZ-diabetic rats treated with sildenafil alone or treated with sildenafil + ALT-711 (Figure 4). However, there was an abundant staining of NFκB-p65 and MAP kinase-p38 in the endothelium of STZ diabetic rats (group 2) compared with the control group and treatment groups (group 3 and 4) as well. Negative control slides demonstrated no staining for NFκB-p65 or MAP kinase-p38 (Figure 4).

Apoptosis using TUNEL assay

Apoptosis of cavernosal cells were abundantly increased in STZ diabetic penises than the control group. Additionally, apoptotic cells in the endothelium as well as in the smooth muscle cells was

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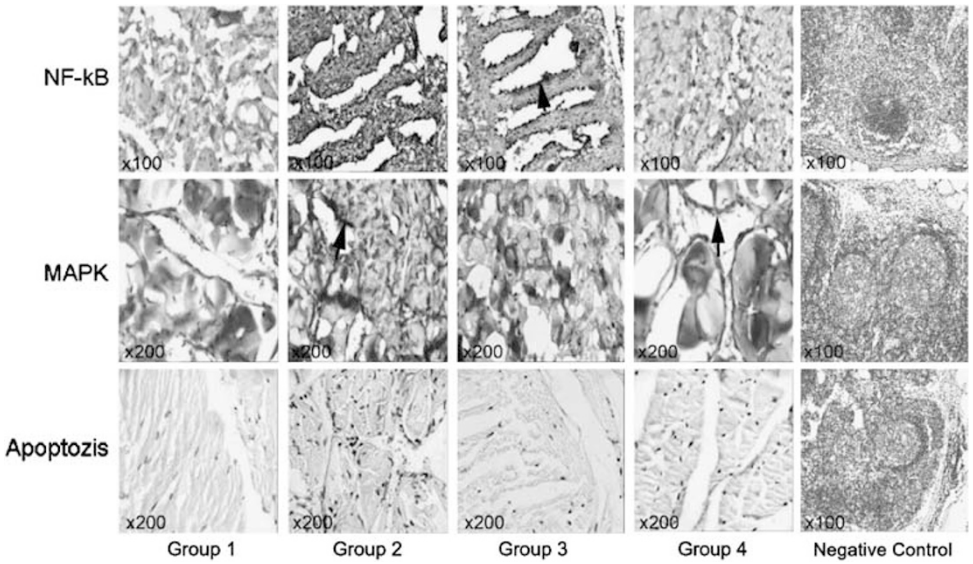


Figure 4. IHC analyses of rat corpus cavernosum for NF-κB, MAP kinase and apoptosis (with TUNEL assay) in all groups. (Arrowheads show strong staining areas). The staining for NF-κB, MAP kinase and apoptosis were more intense in group 2 than the remaining groups. All image magnification includes the lower corner of the image. IHC, immunohistochemical; MAP kinases, mitogen-activated protein kinases; NF-κB, nuclear factor-κB.

lacking in sildenafil-treated and sildenafil + ALT-711-treated rat penises when compared with the diabetic penises (Figure 4). The mean final scores for control, diabetic, diabetic treated with sildenafil alone and diabetic treated with sildenafil + ALT-711 were 1.2, 8.7, 2.6 and 1.4, respectively. Significantly, the scores of apoptosis in the penile tissue in groups 1, 3 and 4 were lower than the score in group 2 ($P<0.05$). In addition, the difference between group 3 and group 4 was statistically significant ($P<0.05$).

In vivo erectile function

Erectile responses to cavernosal nerve stimulation (CNS) are given in Figures 5a and b. The magnitude of the increase in intracavernosal pressure (ICP)/mean arterial pressure (MAP) in response to CNS in the STZ diabetic rats was significantly lower ($P<0.05$) than the control rats, whereas rats treated with sildenafil + ALT-711 had greater responses to CNS (7.5 V setting) that was similar to the response obtained in the control rats (Figure 5a). Furthermore, the ICP values obtained from group 4 was significantly higher than group 3 (7.5 V setting). Additionally, the total ICP (total duration of erection) in the sildenafil-treated STZ diabetic rats was significantly lower than the control rats. However, in the STZ diabetic rats treated with sildenafil + ALT-711, the total ICP level was significantly increased both at the 5 and 7.5 V settings, which was similar to the control group. Additionally, there was also a significant statistical difference between group 3 and group 4 for total ICP levels as shown in Figure 5b.

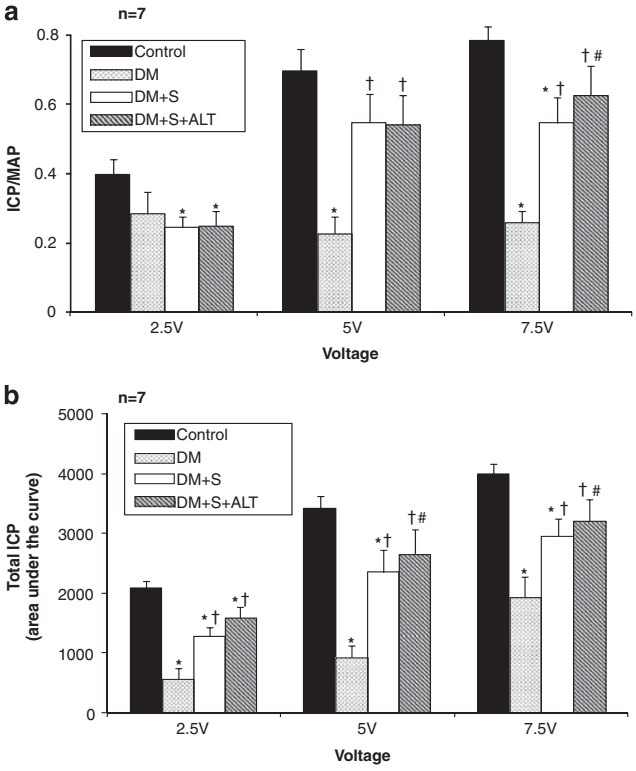


Figure 5. Voltage-dependent erectile response (ICP/MAP) (**a**) and total ICP (area under erectile curve) (**b**). After cavernous nerve stimulation in control, STZ diabetic rats, STZ diabetic rats treated with sildenafil and ALT-711. * indicates $P<0.05$ versus control, † indicates $P<0.05$ versus DM and # indicates $P<0.05$ versus DM + S. DM, diabetes mellitus; ICP, intracavernosal pressure; MAP, mitogen-activated protein; S, sildenafil; STZ, streptozotocin.

DISCUSSION

As shown in several investigations, this study demonstrated that STZ-induced diabetes causes significant ED in rats within 2 months. In the present research we aimed to investigate the effect of a combination therapy (sildenafil + ALT-711) on erectile function in a diabetic rat model. Additionally, different than several previous reports, we have also assessed the effect of chronic administration of sildenafil instead of on demand use on erectile function in the same animal model. Our results showed that diabetic animals treated with a combination of sildenafil + ALT-711 have preserved erectile function, which was almost similar to nondiabetic control animals. Moreover, the combination of sildenafil and ALT-711 in STZ diabetic rats resulted in an increased erectile response that was greater than sildenafil alone. Even though there was not statistically significant difference in ICP responses to CNS between two treatment

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groups (group 3 versus group 4) for 5 V setting; for 7.5 V setting ICP responses were significantly better in group 4 than group 3. Additionally, total ICP levels, which indicate to the total duration of erection, were also found significantly greater in group 4 than in group 3.

Selecting this animal model has two reasons. First, we aimed to assess the effect of a combination therapy namely sildenafil + ALT-711 (cross-link breaker of preformed AGEs-advanced glycation end products) on erectile function in STZ diabetic animals. In our previous studies, we have already shown that the breakdown of preformed AGEs reverses ED in diabetic rats. Furthermore, it has also been revealed that an inhibitor of AGEs have a protective effect on erectile function in STZ diabetic rats.[4–6] Actually, the main goal of the present paper was to determine whether combination of sildenafil + ALT-711 has a greater effect than sildenafil alone on erectile function in STZ diabetic rats or not. Second, we aimed to reveal the effect of that treatment option on several molecules, which are widely accepted as having crucial roles in erectile physiology. Furthermore, we have also investigated the effect of both diabetes and the preferred treatment option on some molecules including NFκB-p65 and MAP kinase-p38, which are possibly related to diabetes-associated ED. Finally, we investigated the apoptotic levels in cavernosal tissues from our different animal groups to broader our understanding about the pathophysiological pathways in diabetes-associated ED.

During the last 2 decades, PDE5 inhibitors have been used as the first-line therapeutic option for the treatment of ED worldwide. In several cohorts, it has been shown that sildenafil is an effective and well-tolerated treatment for ED in men with diabetes.[7,12,15] However, the results of those studies showed that sildenafil does not have the same efficacy in improving erectile function in diabetic men when compared with other ED etiologies. The decreased efficacy of sildenafil in men with diabetes-related ED lead several authors to investigate several other treatment options such as combination treatments and chronic use of PDE5 inhibitors for the treatment of erectile function in that 'hard to treat' population.

Recently, De Young *et al*.[8] have assessed the effect of sildenafil combined with free oxygen radical scavenger namely vitamin E on erectile function in a diabetic rat model. The results of that study suggested that significant increases in ICP were measured in the animals receiving combined vitamin E plus sildenafil treatment when compared with animals either treated with vitamin E or sildenafil alone. The authors concluded that vitamin E enhanced the therapeutic effect of sildenafil, which indicated a potential means of salvaging erectile function among men who are refractory to sildenafil.[8]

Similarly, Bivalacqua *et al*.[12] reported the effect of combination of sildenafil and NO synthase gene therapy on erectile function in STZ diabetic rats. The authors clearly showed that overexpression of eNOS and cGMP in combination with sildenafil significantly increased both the ICP and total ICP to CNS in the STZ diabetic rats, which was similar to the response observed in control rats. More importantly, the total erectile response was greater in STZ diabetic rats receiving eNOS gene therapy plus sildenafil than STZ diabetic rats receiving sildenafil or eNOS gene therapy alone.

Other than the combination therapy options, the chronic administration of sildenafil instead of on demand or single dose therapy have been suggested in several experimental studies for the treatment of diabetes-related ED. Ahn *et al*.[16] have shown that the chronic use of a PDE5 inhibitor namely DA-8159 could attenuate the development of ED in diabetes, and its effect is associated with an improvement in the endothelial function. In a similar study, Park *et al*.[17] reported that chronic treatment with PDE5 inhibitor (SK-3530) activated Akt signaling, which lead to suppression of pro-apoptotic stimuli and maintained erectile function in a rat model of diabetic erectile dysfunction. Furthermore, De Young *et al*.[18] investigated the effect of chronic administration on erectile function in a diabetic rat model. The authors reported that vardenafil was effective in treating diabetic-induced ED with the greatest effect achieved through chronic dosing. Additionally, endothelial cells eNOS protein expression was increased in rats treated with vardenafil in a chronic fashion.

Seftel *et al*.[19] first reported that AGEs are a complex and heteregenous group of compounds, which have been implicated in diabetes-associated complications, and are significantly increased in human diabetic penile tissue. Furthermore, the role of AGEs in DM-related ED has been reported in several experimental studies.[4–6] Recently it has been reported that there are several receptors of AGEs, which mediate intracellular signaling and induce changes in cellular function. Interaction of AGEs with their specific receptors (RAGEs) generate intracellular oxidative stress, which leads in activation of both free radical-sensitive transcription factor (NF-κB) and mitogen activated protein kinases.[20,21] Previously, Nangle *et al*.[22] reported that a MAP kinase inhibitor namely LY2161793 were able to correct NO-dependent indices of diabetic erectile autonomic neuropathy and vasculopathy. Furthermore, recently Angulo *et al*.[23] showed the preventive effect of an antioxidant AC3056 on the diabetes-induced increase of NF-κB levels in penile tissue. In our present study, we investigated the expressions of both molecules in diabetic rat penile tissue. Furthermore, we have revealed that the increased expression of those molecules was decreased in diabetic cavernosal tissues obtained from diabetic rats treated with sildenafil and with sildenafil + ALT-711 as well. Finally, in accordance with previously reported studies, we observed also that although apoptosis was increased in diabetic cavernosal penises, both sildenafil and sildenafil + ALT-711 treatments prevent the apoptotic effect of diabetes in cavernosal tissue.[24,25]

In our study, 5-HMF and MDA were indicators for penile tissue AGEs and oxidative stress, respectively. Both parameters were increased in diabetic penile tissue, whereas they were both decreased after sildenafil + ALT-711 treatment. However, the most significant decrease of these parameters were observed in the combination group, which supported our hypothesis that combination therapy have more beneficial effect than sildenafil alone in the treatment of diabetes-related ED. Similar results have been shown for the penile tissue iNOS levels. In our study, we demonstrated that whereas iNOS levels were significantly increased in diabetic penises, it was significantly decreased in animals either treated with sildenafil or sildenafil + ALT-711. Several previously reported studies have shown that iNOS levels are significantly increased in diabetic penile tissue as a response to inflammatory reactions.[4] Furthermore, in a recent paper, Gonzales-Cadavid and Rajfer [26] speculated that iNOS would protect erectile tissue by counteracting fibrosis that impairs cavernosal smooth muscle relaxation. Additionally, they reported that further studies are needed to conclusively evaluate the possible effect of iNOS on erectile physiology. Moreover, whereas cavernous cGMP content was preserved in penile tissue of diabetic rats that underwent combination treatment, in diabetic animals treated with sildenafil alone cGMP level was significantly decreased when compared with the control and sildenafil + ALT-711-treated diabetic animals as well. In our study, we investigated the basal cGMP levels, which could be considered as an index of tissue health (for example, endothelium/nerve/smooth muscle function). Although the ideal way of cGMP measurement is to assess the cGMP levels during CNS, to our knowledge at least nowadays it is very difficult to perform such a technique related to that matter. In contrast with those results, even though penile tissue eNOS protein expression was higher in the STZ diabetic rats treated with ALT-711 + sildenafil than the levels observed in STZ diabetic animals,

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the most abundant expression was observed in animals that were treated with sildenafil alone. Actually, we were not able to explain this discordance, however it may depend on the relatively small numbers of penile tissue samples, which made the statistical analyses a little bit confusing. Owing to technical reasons, we were not able to examine eNOS phosphorylation. However, we have to express here that measurement of eNOS phosphorylation makes more sense than eNOS protein measurement alone. We believe that further studies with large number of penile tissue samples as well as eNOS phosphorylation measurement will help to overcome such limitations.

Another limitation that we need to discuss is the time point of treatment with sildenafil alone or sildenafil + ALT-711 in the present study. Recently, it has been reported by Cellek and Usta that at least a 6–8-weeks diabetes period is required to observe any irreversible neural degeneration in penile tissue.[6,27,28] Hence, to assess the protective effect of any treatment option, the ideal way is to start the mentioned ED treatment options at least after a 6–8-weeks diabetes period. We should express that as the STZ diabetes model used in the present study is a typical type-1 diabetes model, it is very difficult to keep the animals alive for such a long time period (6–8 weeks for diabetes and an additional 6–8 weeks for treatment). However, the STZ diabetes model have been preferred and suggested by several authors in the literature. Contrarily, we strongly believe that in such studies the ideal model is to use the genetically type-2 diabetes-induced animals.

As mentioned, in our present study we have performed the sildenafil treatment in a chronic fashion. However, some authors may conclude that we could have stopped the sildenafil therapy 72 h before the erectile function assessment to say whether sildenafil would be able to everse the pathology, and not only the erectile function. Another important issue is that because of technical reasons, we were not able to measure the penile tissue nNOS expression, which is the most important source of NO in the penis. Finally, in our present study we have not established a diabetic group of animals treated only with ALT-711. The reasons for that were: first, the main goal of the present study was to assess the effect of a combination treatment namely sildenafil + ALT-711 and compare it with sildenafil alone. Second, we thought that it is really difficult to establish five different groups of animals and compare the statistical results of several parameters in such a busy study group. Moreover, the therapeutic effect of ALT-711 on erectile function in diabetic rats has been show in one of our previous studies. The mentioned study clearly showed that treatment with ALT-711 but not with an AGE inhibitor aminoguanidine reversed ED and nNOS depletion, and reduced serum and penile tissue AGE levels. Although it is very difficult to compare the results obtained from the mentioned study with our findings, if we look at the results of both studies we can conclude that ALT-711 alone has a robust effect on erectile function, which seems at least better than sildenafil alone. However, in our previous investigation we have assessed the the penile tissue nNOS content, while in the present investigation the eNOS and iNOS expression, so it is not convenient to make any comparison related to the effect of ALT-711 versus sildenafil treatment groups on these mediators, involved in erectile physiology. Furthermore, in our previously reported study, we performed qualitative measurements of penile tissue AGE levels, whereas in the present manuscript we investigated the IHC presence of AGE-related molecules such as NF-κB and MAP kinase, which also makes the comparison between ALT-711 and sildenafil treatments pretty difficult. Altogether, those reasons suggest us to compare the therapeutic effect of both ALT-711 and sildenafil alone on erectile function in STZ diabetic rats in a further study. That would absolutely broaden our understanding about the possible synergistic/additive effect between ALT-711 and sildenafil.

Conclusions

Even though there are several recent investigations the exact pathophysiology of diabetes-related ED has not been defined yet. In our present study, we have shown that combination treatment options such as sildenafil + ALT-711 seem more efficient than sildenafil alone in the treatment of diabetes-related ED. The effect may occur over several molecules that have been suggested to have crucial roles in both erectile physiology and diabetes-related erectile dysfunction. Furthermore, some of the results of the present study showed that some molecules, previously reported to have critical roles in vascular pathologies such as NF-κB and MAP kinase, are also involved in diabetes-related ED. Altogether, these data may broaden our understanding about diabetes-related ED and alternative treatment options for 'hard to treat' populations.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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