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의학박사 학위논문

**Effects of the start time of glycemic control
on erectile function in streptozotocin-induced
diabetic rats**

**Streptozotocin 유도 당뇨병 백서에서
혈당 조절의 시작 시기가 발기력에 미치는 영향**

2017 년 2 월

서울대학교 대학원
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ABSTRACT

Effects of the start time of glycemic control on erectile function in streptozotocin-induced diabetic rats

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Introduction : Diabetes-induced erectile dysfunction (DMED) correlates with diabetes duration and glycemic control. Strict glycemic control can improve DMED to near-normal status. This study evaluated the effect of glycemic control start time on erectile function in streptozotocin-induced diabetic rats.

Materials and Methods: Eight-week-old Sprague-Dawley rats were divided into normal controls (group C); untreated diabetic rats (group DM); and rats treated after 7 weeks (group 7W), and 10 weeks (group 10W) from DM. Streptozotocin was injected to induce diabetes. Treated diabetic rats received a timed daily injection of insulin. After 14 weeks of lab-controlled diabetes, erectile function was assessed by cavernous nerve stimulation. And an evaluation of histological and biochemical markers from corporal tissue was performed.

Results: Group DM showed the ratio of intracavernosal pressure to mean arterial pressure significantly lower than groups C, 7W, and 10W (10W vs. DM, $p < 0.001$). Groups 7W and 10W responded similarly ($p = 0.170$, 20 Hz; $p = 0.769$, 10 Hz) but did not recover to group C's level (group C vs. 7W, $p < 0.001$). The percentage of α -smooth muscle actin increased

more with earlier start times, and group DM's percentages decreased significantly (group 10W vs. DM, $p < 0.001$). Apoptotic cell density recovered significantly only in group 7W, comparable with group C (group 7W vs. 10W, $p = 0.020$). Densitometry analysis showed molecular changes in cavernosal tissues. As start times became earlier, for all molecules (eNOS, Akt, MYPT1, and PECAM-1), treatment groups' results neared those of group C.

Conclusion: When diabetic treatment started earlier, diabetic rats recovered erectile function similar to controls, but not to normal levels. The level of glycemic control was expected to be more important than the start time of DM treatment.

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Keywords: Erectile dysfunction; Diabetes Mellitus; Animal model of diabetes

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INTRODUCTION

Diabetes mellitus (DM) is known as a major cause of erectile dysfunction (ED), and poor glycemic control is closely related to severe ED.¹⁻³ In the Massachusetts Male Aging Study, diabetes patients reportedly have a three times greater risk of complete ED compared with nondiabetic patients (28% vs 9.6%).⁴ And ED occurs 10 to 15 years earlier in men with diabetes than men of general population.⁵ In addition, ED affects up to 75% of men who have DM and impairs quality of life.⁶ Moreover, the risk of diabetes-induced ED (DMED) is strongly related to glycemic control, duration of DM, and the presence of diabetic complications.⁷ Phosphodiesterase type 5 inhibitors (PDE5Is) are less effective for treating DMED than ED from other causes.⁸ Approximately half of patients with DMED respond to oral PDE5Is. Deficiency of nitric oxide (NO) dependent cavernosal smooth muscle relaxation and decreased guanyl cyclase activity may be causes of the reduced response rate to PDE5Is.⁸

DM may induce ED by a number of pathophysiological changes influencing psychological function, central nerve system function, androgen secretion, peripheral nerve activity, endothelial cell function, and smooth muscle contractility.⁹ Penile erection is known as a hemodynamic process which is managed by penile smooth muscle tone. Neuronal and endothelial NO induces 3',5'-cyclic guanosine monophosphate and this results in deactivation of myosin light chain. This finally causes relaxation of cavernosal smooth muscle.¹⁰ And Rho kinase pathway inhibits myosin light chain phosphatase against NO activity, so maintains the contraction of corporal smooth muscle.¹¹ Endothelial dysfunction is considered as a potential principal mechanism of DMED.¹²⁻¹⁴ NO dependent endothelial dysfunction occurs early in diabetes, followed by structural changes in the corpus cavernosum and autonomic neuropathy.¹⁵ DMED can be explained as a result of impairment of NO synthase (NOS) activity and downregulation

of the mediators from NO, such as cyclic guanosine monophosphate, in the corpus cavernosum. The changes of impaired endothelial function in the diabetic penis include reduced endothelial NOS (eNOS) expression, decreased eNOS activity, impaired eNOS phosphorylation, increased oxidative stress, and increased activity of the Rho kinase signaling pathway.¹⁰ And in the diabetic rats, inhibition of Rho kinase pathway improved eNOS protein content and activity thus restoring erectile function.¹⁶ Also Rho kinase expression has significantly increased in penile tissues of streptozotocin-induced diabetic rats.^{17,18} As a result, the response to PDE5Is decreases and is compromised long term.²

In our previous study, time-dependent changes in erectile function and responses to PDE5Is were investigated in streptozotocin-induced diabetic rats.² Erectile function was assessed at 6, 8, 10, 12, and 14 weeks after DM induction in both diabetic rats and age-matched control rats. After intracavernosal pressure (ICP) was collected and returned to normal baseline value, animals received intravenous administration of DA-8159, a novel PDE5I. And cavernous nerve stimulation was repeated 3 minutes later after the injection. Compared with age-matched controls, no evidence of impairment in the erectile function was observed until 6 weeks after induction of DM. After 8 weeks of DM induction, erectile function began to deteriorate, but there were no differences between diabetic and control rats after intravenous injection of PDE5I. In comparison with normal rats, 10-week diabetic rats showed significant decrease in erectile function, but after administration of PDE5I, erectile responses were significantly increased up to normal control values at high frequency (20 Hz) stimulation. However, after 12 and 14 weeks of diabetes, erectile responses were impaired remarkably, and could not be normalized even after administration of PDE5I. In the Western blot analysis, eNOS activity significantly decreased after 6 weeks of diabetes, and Rho kinase expression remarkably increased after 8 weeks of diabetes. And apoptotic

index in diabetic rat cavernosum was significantly increased at 10 weeks after induction of diabetes. Therefore, starting DM treatment before erectile function response is compromised may be the ideal strategy.

Glycemic control is the primary treatment for preventing and relieving DMED.^{3,19} Romeo and collaborators reported that, in DM patients, erectile function decreased as glycosylated hemoglobin (HbA1c) increased and that ED was associated with degree of glycemic control.¹⁹ In a previous animal study, investigators tried to evaluate time-dependent changes in erectile function and pathophysiology after induction of diabetes without insulin treatment.² Over the course of diabetic induction, impairment of erectile function was followed by decreased responsiveness to PDE5I at a certain point and activation of Rho kinase pathway played an essential role on it.² But, only PDE5I treatment without glycemic control is not applicable in real practice, because glucose levels of diabetic patients are controlled to some degree. In another animal study, strict glycemic control recovered erectile function close to normal levels in diabetic rats.³ Rats were divided into four subgroups: normal control, diabetes with multiple insulin injections, diabetes with a single insulin injection, and untreated diabetes. And insulin treatment was started at 10 weeks after DM induction, which was considered as the point of overt ED development. After 4 weeks of insulin treatment, erectile function of diabetic rats with multiple insulin injections showed near normal recovery from untreated diabetic rats, but that of diabetic rats with a single insulin injection showed only partial recovery. And the apoptotic index increased and smooth muscle components were diminished with poor glycemic control. Western blot analysis showed activation of Rho kinase pathway and deactivation of NO pathway in diabetic rats. Insulin treatment improves hyperglycemic conditions and enhances activation of the phosphatidylinositol 3-kinase-dependent insulin-signaling pathways which regulate endothelial production of NO crucial for erectile function.²⁰

However, after irreversible impairment of erectile function, the effects of glycemic control were limited; thus, the start time of glycemic control may be critical to recovering erectile function. Cellek and collaborators suggested that, in diabetic rats, the process by which nitrenergic neurons innervating the penis degenerate consisted of two phases.²¹ In the first phase, nitrenergic nerve fibers lost some of their neuronal NOS (nNOS) content and function. In the second phase, nitrenergic degeneration occurred in the cell bodies in the ganglia, deteriorating nitrenergic function completely. The functional changes of first phase were reversible with insulin treatment; however, in the second phase, neurodegeneration caused by apoptotic cell death was irreversible.²¹ However, Cellek did not evaluate erectile function, so functional response in DMED was not clearly elucidated. In addition, our previous study showed that overt DMED was started from 10 weeks after DM induction, and response of PDE5Is was lost at this time period.²

Several studies were previously performed to evaluate the recovery of erectile function and changes of cavernosal structure with diabetic rats.^{2,3,12,14,17,18,21} However, no previous study has evaluated erectile functional response at different start time of glycemic control in DMED. Therefore, the aim of this study was to evaluate the effects of glycemic control start time on erectile function in streptozotocin-induced diabetic rats.

MATERIALS AND METHODS

Experimental Animals and Study Design

This study was carried out in strict accordance with the recommendations in the *Guide for Care and Use of Laboratory Animals* of the National Institutes of Health. The protocol for all experiments was approved by the Institutional Animal Care and Use Committee of Seoul National University Hospital (IACUC approval no.: 13-0138-C2A1). Eight-week-old Sprague-Dawley rats (N = 60) were used in this study. Rats were randomly divided into four subgroups (N = 15 in each group): normal controls (C); those with diabetes treated after 7 weeks (7W) and 10 weeks (10W); and those with untreated DM. All rats weighed 260-310 g before inducing DM. After overnight fasting, a single intraperitoneal injection of 60 mg/kg streptozotocin with a citrate phosphate buffer (50 mM sodium citrate, pH 4.5) was injected to induce DM. The same volume of citrate buffer was administered to the normal controls. DM was confirmed when blood glucose levels exceeded 300 mg/dL after 48 hours of streptozotocin administration. All rats except 6 developed DM after a single injection of streptozotocin, and non-hyperglycemic rats received another injection of 60 mg/dL streptozotocin. Within 3 days, every rat had developed DM. Blood glucose levels were measured using a GlucoDr Plus[®] Glucometer (AGM-2300, Almedicus, Gyeonggi-do, Korea). All rats in diabetic condition showed polydipsia, polyphagia, and hyperuresis.

During untreated period, body weight and blood glucose levels were monitored once a week. According to protocols, in treatment groups 7W and 10W, daily single insulin injections were administered subcutaneously until week 14 after inducing DM. Following the initial dose of 5-6 units of neutral protamine Hagedorn (NPH), doses were adjusted based on blood glucose levels, checked 3 times per week. Glycemic control continued similar to that in a previous study.³ If blood glucose level was over 300

mg/dl, the insulin dose was elevated by 2 units, and if blood glucose level was under 150 mg/dl, the dose was lowered by 1 unit. This method of insulin adjustment was applied to achieve the level of glucose control between strict and poor control. Also the target level of glycemic control was a range of 6.0–7.0% of HbA1c level, which was result of 4 week insulin treatment. The start time of treatment was decided based on previous study data using the same animal model.² In group 10W, insulin was injected after 10 weeks of diabetic induction, timed to induce overt ED. In the early treatment group (7W), insulin was administered after 7 weeks of DM induction, timed to precede deterioration of erectile function.

In vivo Assessment of Erectile Function

After 14 weeks since DM induction, erectile function was assessed under anesthesia using the technique described in previous studies.^{2,3,22,23} Rats were anesthetized with Zoletil of 50 mg/kg and a 24-gauge angiocatheter was then introduced into the carotid artery. And the corpus cavernosum was then introduced into the carotid artery. And the corpus cavernosum was exposed and cannulated with a 24-gauge butterfly needle. Mean arterial pressure(MAP) was continuously monitored via the carotid artery, and ICP was continuously monitored via the corpus cavernosum. The major pelvic ganglion and cavernous nerve were revealed at the lateral side of the prostate, and a bipolar electrode was connected to an electrical stimulator (S48, Grass Instruments, Quincy, MA, USA). Electrical stimulation of 2 V was performed for 30 seconds at 0.2-millisecond intervals with frequencies of 10 (submaximal) and 20 (maximal) Hz, respectively. The intervals between each stimulation lasted approximately 3 minutes. Data were collected electronically at every stimulation (PowerLab, ADInstruments, Colorado Springs, CO, USA), and the ratios of ICP to MAP, and area under curve (AUC) to MAP, were calculated. Authors measured the curve for 5 minutes to calculate AUC from the beginning. A total of 8 ml whole blood was drawn from the carotid arteries

to measure the percentage of HbA1c (Roche Diagnostics, Indianapolis, IN, USA).

In vitro Assessment of Structural Change

After completing functional studies, the cavernosal tissue was removed intact. The middle part was kept overnight in 10% formaldehyde solution and embedded for histological evaluation. Remnant tissue was promptly placed in liquefied nitrogen and stored at -80°C .

Apoptotic index was measured using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method, as previously reported.³ After deparaffinization and rehydration, sections of 5-mm thickness were incubated with 20 mg/ml Proteinase K at 37°C for 30 minutes and quenched with 3% hydrogen peroxide in methanol at room temperature for 5 minutes. The sections were then incubated with a terminal deoxynucleotidyl transferase (TdT) enzyme, followed by incubation with antidigoxigenin-peroxidase conjugate. Apoptotic cells were counted by identifying all double-stained nuclei with fluorescein. Slides were reviewed with a LSM 510 META NLO confocal microscope (Carl Zeiss, Jena, Germany). In each slide, entire cavernosal tissue was captured under magnification fields ($\times 100$). The apoptotic index represented the ratio of apoptotic cells to total cells in the given area, calculated using ImageJ analysis software (National Institutes of Health, Bethesda, MD, USA). Results were expressed as apoptotic cell density, calculated as the number of apoptotic cells/ mm^2 of cavernosal surface area.

Alpha-smooth muscle actin (α -SMA) was immunohistochemically stained to assess the percentage of the smooth muscle cell component (% α -SMA).³ After fixing and embedding the penile tissue, sections were deparaffinized and rehydrated. Antigens were retrieved and endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol and nonspecific binding was inhibited. Specimens were incubated overnight

in primary anti- α -SMA antibody (1:100; Dako, Glostrup, Denmark) and incubated with a biotinylated secondary antibody (1:2,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for counterstaining. The antigen-antibody reactions were developed by diaminobenzidine solution. The ratio of positive staining of α -SMA to total area was calculated under magnification $\times 25$ in four duplicate sample sections using Image-Pro Plus 4.5 software (Media Cybernetics, Silver Spring, MD, USA).

Western Blot Analysis

Key molecules associated with NO and Rho kinase pathways were investigated using Western blot analysis.^{3,22} Equal amounts of protein extracts (20–50 μ g) from penile tissue were separated in sodium dodecyl sulfate polyacrylamide gels, transferred to nitrocellulose membranes. Samples were incubated overnight with several primary antibodies: anti-eNOS (1:3,000, BD Biosciences, San Jose, CA, USA); anti-phospho-eNOS (Ser1177, 1:1,000, Cell Signaling Technology); anti-myosin phosphatase target subunit1 (MYPT1) (1:2,000, Cell Signaling Technology); anti-phospho-MYPT1 (Thr696, 1:1,000, Millipore, Charlottesville, VA, USA); anti-Akt (1:5,000, Cell Signaling Technology); and anti-phospho-Akt (Ser473, 1:1,000, Cell Signaling Technology). CD31 antibody (PECAM-1) (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to evaluate cavernosal endothelial cells. Bound antibodies were detected using peroxidase-conjugated anti-rabbit immunoglobulin G antibodies and the enhanced chemiluminescence system (GE Healthcare, Piscataway, NJ, USA). To adjust for loading differences, the membranes were reprobbed with monoclonal anti- β -actin antibodies. Results were quantified by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis

Data are shown as mean \pm standard deviation. Differences in blood glucose levels and erectile parameters were evaluated in relation to start time of insulin treatment. Among more than three groups, analysis of variance (ANOVA) with post-hoc Duncan's analyses was used. A two-tailed *P* value of < 0.05 was considered statistically significant for all analyses, which were performed using SPSS[®] version 22.0 software (IBM, Armonk, New York, USA).

RESULTS

Levels of Glycemic Control

Rats in diabetic groups DM, 7W, and 10W had higher blood glucose levels than controls (Table 1, Figure 1) and reached at 900 mg/dl of maximum measurement value. After insulin treatment, glucose levels in the 7W and 10W groups started to decrease until they were intermediate between those of groups C and DM. HbA1c level of diabetic groups (7W, 10W, and DM) were significantly higher than that of group C. And the HbA1c levels of the two groups treated with insulin (7W and 10W) were significantly lower than non-treated group DM (10W vs. DM, $P < 0.001$), but there was no difference between treatment group 7W and 10W ($P = 0.420$) (Figure 2). Body weights in the diabetic groups decreased after inducing DM but trended upward in groups treated with insulin (Table 2, Figure 3).

Erectile Function Assessment

The baseline ICP/MAP ratios in the group C, 7W, 10W, and DM were 0.11 ± 0.03 , 0.11 ± 0.04 , 0.12 ± 0.03 , and 0.13 ± 0.04 , respectively ($P = 0.315$) in 20 Hz and 0.11 ± 0.05 , 0.10 ± 0.03 , 0.11 ± 0.03 , and 0.13 ± 0.04 , respectively ($P = 0.289$) in 10 Hz. Figure 4a compares ICP/MAP among experimental groups (Table 3). Group DM's responses significantly decreased compared with group C (0.35 ± 0.05 vs. 0.84 ± 0.07 , $P < 0.001$ in 20 Hz; 0.27 ± 0.07 vs. 0.81 ± 0.09 , $P < 0.001$ in 10 Hz). Although groups 7W and 10W showed better erectile function recovery than group DM (10W vs. DM, 0.66 ± 0.13 vs. 0.35 ± 0.05 , $P < 0.001$ in 20 Hz; 0.59 ± 0.15 vs. 0.27 ± 0.07 , $P < 0.001$ in 10 Hz), the responses of group 7W did not reach those of the normal control group C (0.72 ± 0.05 vs. 0.84 ± 0.07 , $P < 0.001$ in 20 Hz; 0.60 ± 0.08 vs. 0.81 ± 0.09 , $P < 0.001$ in 10 Hz). No differences emerged between groups 7W and 10W (0.72 ± 0.05 vs. 0.66 ± 0.13 , $P = 0.521$ in 20 Hz; 0.60 ± 0.08 vs.

0.59 ± 0.15, $P = 1.000$ in 10 Hz), but group 7W showed increased response than group 10W. Figure 4b displays the results for AUC/MAP (Table 4), whose trends resemble the results for ICP/MAP.

Structural Changes According to Treatment

α-SMA percentage decreased significantly in group DM compared with group C (3.95 ± 0.86 vs. 7.81 ± 2.03, $P < 0.001$) (Table 5, Figure 5). Treated groups 7W and 10W had significantly higher % α-SMA than group DM (10W vs. DM, 7.05 ± 1.71 vs. 3.95 ± 0.86, $P < 0.001$). No significant differences in % α-SMA appeared among groups C, 7W, and 10W ($P = 0.576$). Apoptotic cells significantly increased in group DM compared with group C (29.23 ± 18.46 vs. 9.26 ± 10.48, $P < 0.001$) (Table 6, Figure 6). Apoptotic cell density did not increase in group 7W compared with group C (11.43 ± 5.41 vs. 9.26 ± 10.48, $P = 0.553$). In addition, apoptotic cell density increased significantly in group 10W compared with group 7W (21.09 ± 15.52 vs. 11.43 ± 5.41, $P = 0.027$), and showed similar results between groups 10W and DM (21.09 ± 15.52 vs. 29.23 ± 18.46, $P = 0.264$).

Western Blot Analysis

Densitometric analysis showed molecular changes in cavernosal tissues (Table 7). The ratio of phospho-/total MYPT1 expression, which is a marker of Rho kinase pathway activation, significantly increased in group DM compared with those of normal and treated groups (Figure 7). And the ratios of phospho-/total eNOS (Figure 8) and Akt (Figure 9), indicators of NO pathway activation, were significantly lower in group DM than group C (eNOS, $P = 0.020$; Akt, $P = 0.008$). Rats treated with insulin showed no differences in MYPT1 ($P = 0.774$), eNOS ($P = 0.605$), and Akt ($P = 0.342$) between group 7W and 10W. In addition, there were no differences in expressions of MYPT1 ($P = 0.625$), eNOS ($P = 0.170$), and Akt ($P =$

0.290) among groups C, 7W, and 10W. However, the densitometric results of treated rats tended to increase with earlier glycemic-control start times. The results of PECAM-1, which showed a marked decrease in CD31-positive endothelial content in cavernosal tissue in group DM than in group C ($P < 0.001$, Figure 10). PECAM-1 increased along with earlier glycemic control start time, and no differences were seen in groups C, 7W, and 10W ($P = 0.075$).

Table 1. Mean blood glucose levels of each group during the experimental period after inducing diabetes

Weeks after diabetes induction	0W	1W	2W	3W	4W	5W	6W	7W
group C	91.4	86.1	91.3	97.2	96.5	101.1	95.8	92.9
group 7W	494.5	696.5	797.5	821.4	877.5	856.6	889.9	885
group 10W	500.2	732.3	794.8	877.3	891.9	893.7	900	900
group DM	386.1	489.5	810.3	760.1	862.3	884.8	900	900

Weeks after diabetes induction	8W	9W	10W	11W	12W	13W	14W	15W
group C	91.9	93.1	87.3	86.2	90.6	92.1	89.9	88.4
group 7W	796.7	526.9	454	423.4	388.2	362.9	329.9	331.7
group 10W	900	898.9	889	800.7	461.9	442.1	399.4	420.4
group DM	893	871.8	899.5	894.1	888.6	889.3	893.5	898

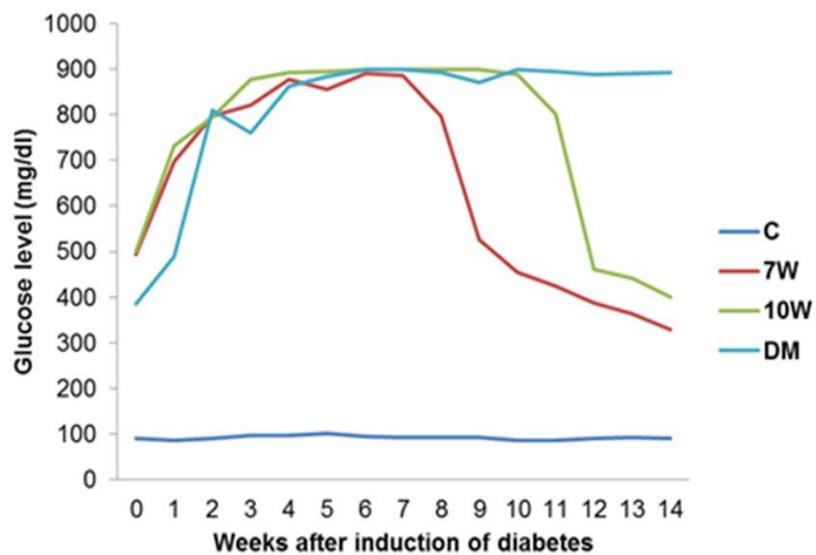


Figure 1. Mean blood glucose levels of each group during the experimental period after induction of diabetes.

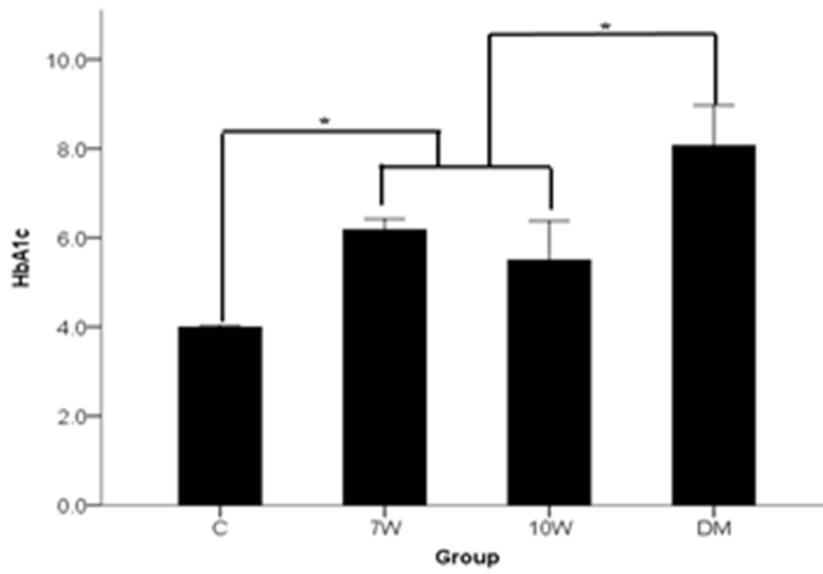


Figure 2. Glycosylated hemoglobin (HbA1c) of each group. * $P < 0.05$.

Table 2. Mean body weights of each group during the experimental period after inducing diabetes

Weeks after diabetes induction	0W	1W	2W	3W	4W	5W	6W	7W
group C	285.4	306.5	338.1	359.7	377.5	393.3	407.6	416.7
group 7W	281.3	259.5	261.7	256.2	249.1	243.9	243.5	240.9
group 10W	281.7	248.6	240.8	227.9	218.2	209.4	204.6	199.2
group DM	286.0	227.8	228.0	222.9	215.6	208.2	198.9	191.8

Weeks after diabetes induction	8W	9W	10W	11W	12W	13W	14W	15W
group C	428.9	439.4	447.7	452.2	460.1	459.8	468.9	476.9
group 7W	227.8	274.8	321.4	346.7	367.6	378.8	389.7	409.6
group 10W	196.7	193.4	189.1	186.1	228.2	274.0	308.5	341.7
group DM	190.6	187.9	187.3	184.6	184.2	184.2	182.0	182.2

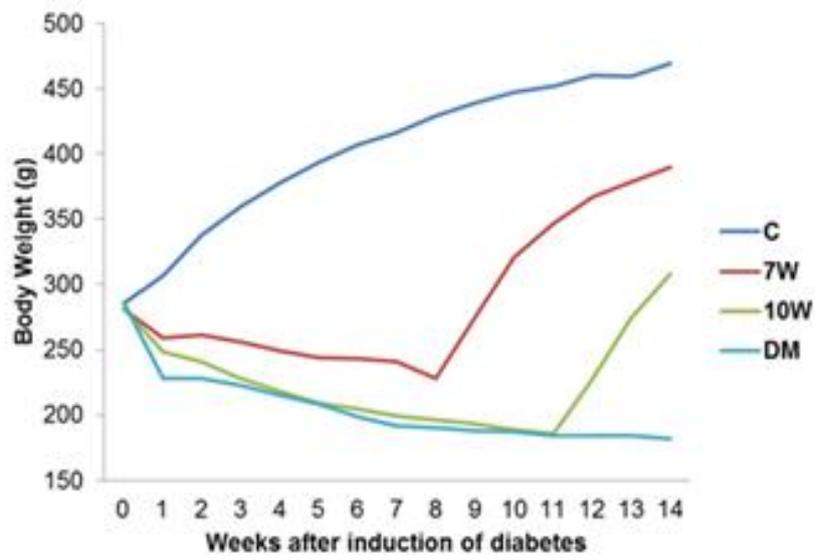


Figure 3. Body weights of each group during the experimental period after induction of diabetes.

Table 3. Mean value of intracavernosal pressure (ICP) / mean arterial pressure (MAP) of each group at 14 weeks after diabetes induction

Intensity of Stimulation	Mean \pm SD	95% CI	Range
10 Hz			
group C	0.805 \pm 0.093	0.760 - 0.850	0.669 - 0.983
group 7W	0.603 \pm 0.076	0.555 - 0.651	0.459 - 0.740
group 10W	0.588 \pm 0.149	0.482 - 0.695	0.381 - 0.894
group DM	0.268 \pm 0.065	0.221 - 0.314	0.123 - 0.359
20 Hz			
group C	0.842 \pm 0.066	0.810 - 0.874	0.749 - 0.980
group 7W	0.718 \pm 0.049	0.686 - 0.749	0.613 - 0.782
group 10W	0.660 \pm 0.130	0.566 - 0.753	0.430 - 0.900
group DM	0.345 \pm 0.054	0.309 - 0.381	0.225 - 0.409

Table 4. Mean value of area under curve (AUC) / mean arterial pressure (MAP) of each group at 14 weeks after diabetes induction

Intensity of Stimulation	Mean \pm SD	95% CI	Range
10 Hz			
group C	22.095 \pm 6.486	18.969 - 25.221	16.040 - 40.140
group 7W	13.366 \pm 3.289	11.276 - 15.456	8.128 - 19.474
group 10W	12.075 \pm 5.823	7.910 - 16.241	7.449 - 15.105
group DM	2.737 \pm 1.732	1.497 - 3.976	1.197 - 6.871
20 Hz			
group C	26.150 \pm 6.955	22.797 - 29.502	19.697 - 46.237
group 7W	17.347 \pm 2.724	15.617 - 19.078	12.926 - 19.758
group 10W	14.761 \pm 6.402	10.181 - 19.340	9.608 - 19.450
group DM	5.099 \pm 2.130	3.669 - 6.530	2.273 - 8.107

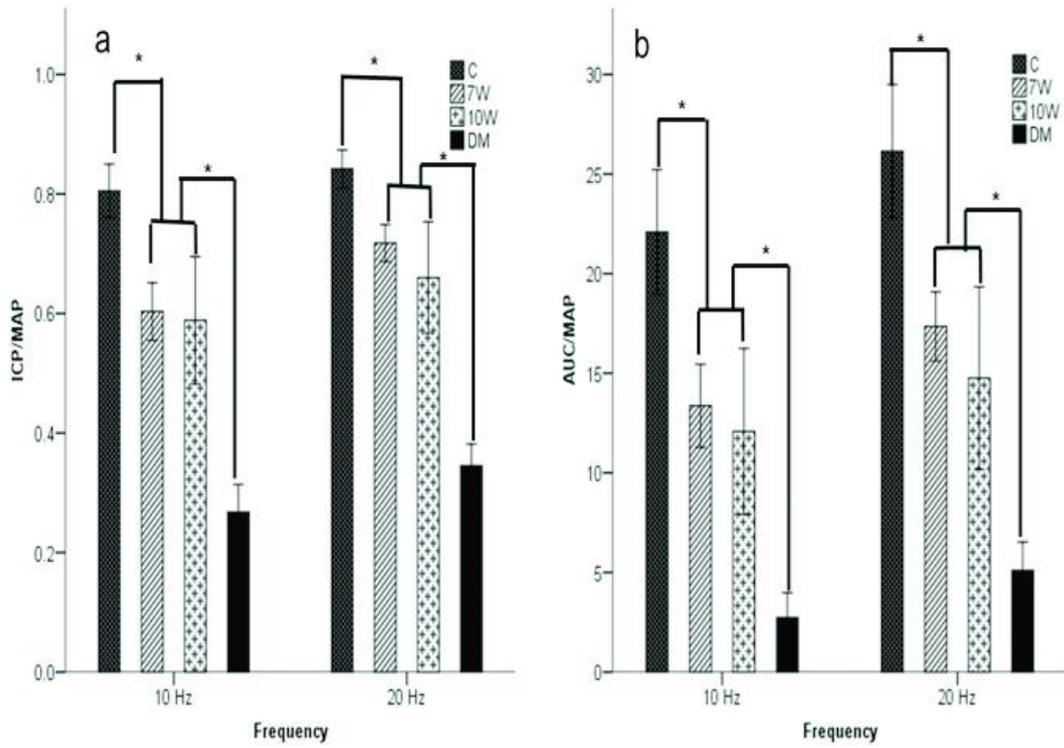


Figure 4. Comparison of erectile parameters for each study group using 10 and 20 Hz frequency stimulation. (a) The ratios of ICP to MAP and (b) the ratios of AUC to MAP for all groups. $*P < 0.05$. ICP, intracavernosal pressure; AUC, area under the curve; MAP, mean arterial pressure.

Table 5. Alpha-smooth muscle actin (α -SMA) positive area (%) of each group

Groups	Mean \pm SD	95% CI	Range
group C	7.806 \pm 2.031	7.138 - 8.474	4.4 - 12.8
group 7W	7.275 \pm 1.798	6.548 - 8.001	4.0 - 11.0
group 10W	7.047 \pm 1.714	6.194 - 7.899	3.1 - 9.4
group DM	3.951 \pm 0.864	3.586 - 4.316	2.1 - 5.6

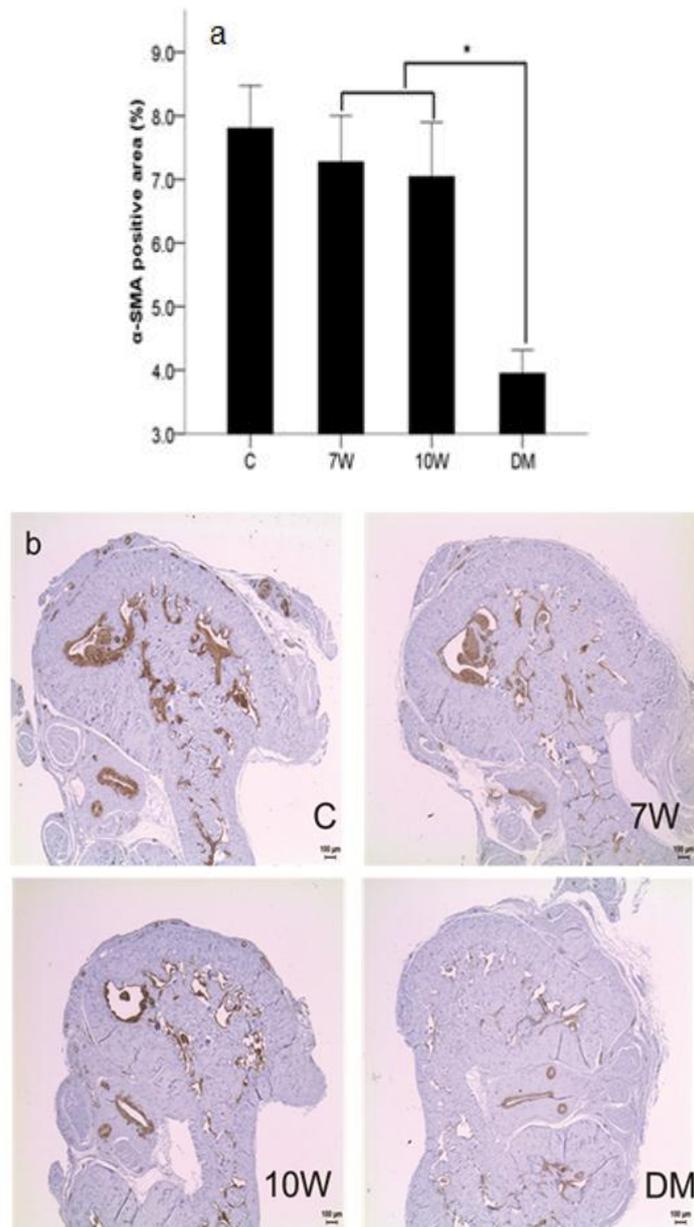


Figure 5. Alpha-smooth muscle actin (α -SMA) expression using immunohistochemical analysis in penile tissue sections. (a) The percentage of smooth muscle cell component (% α -SMA) in each group. (b) Representative images of α -SMA immunohistochemical staining in penile sections. Smooth muscle components appear red (original magnification $\times 25$, scale bar = 100 μm). * $P < 0.05$.

Table 6. Apoptotic cell density (cells/mm²) of each group

Groups	Mean \pm SD	95% CI	Range
group C	9.257 \pm 10.476	5.814 - 12.701	0.9 - 37.7
group 7W	11.427 \pm 5.412	9.240 - 13.613	3.1 - 25.2
group 10W	21.085 \pm 15.523	13.365 - 28.804	2.6 - 65.8
group DM	29.228 \pm 18.463	21.432 - 37.024	6.1 - 88.4

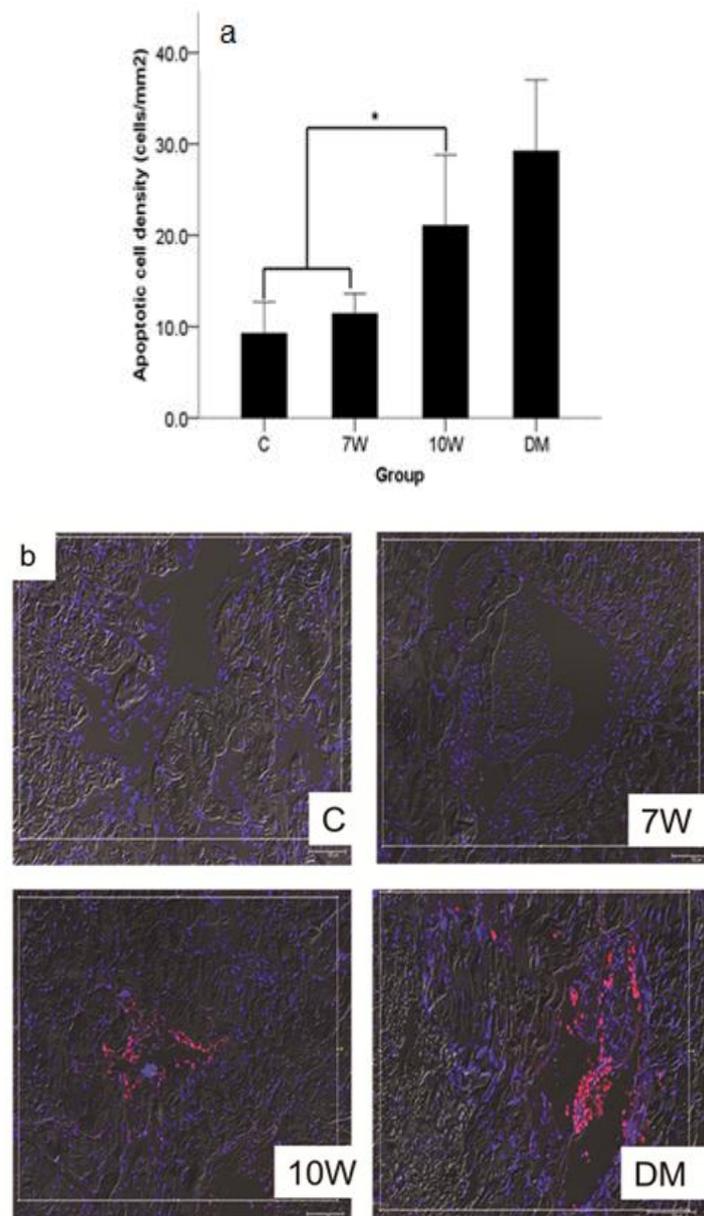


Figure 6. (a) Comparison of apoptotic indices across the groups. Results are expressed as apoptotic cell density (cells/mm²). (b) Apoptotic activity detected by in situ fluorescein labeling of DNA fragments using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) (original magnification x100, scale bar = 100 μ m). * $P < 0.05$.

Table 7. Results of Western blot analysis

Molecules	Mean \pm SD	95% CI	Range
p-MYPT1/MYPT1			
group C	0.659 \pm 0.342	0.414 - 0.904	0.209 - 1.312
group 7W	0.868 \pm 0.635	0.380 - 1.356	0.198 - 2.075
group 10W	0.855 \pm 0.538	0.291 - 1.420	0.149 - 1.231
group DM	1.283 \pm 0.987	0.457 - 2.108	0.362 - 2.980
p-eNOS/eNOS			
group C	3.412 \pm 2.402	1.886 - 4.938	0.517 - 8.644
group 7W	2.177 \pm 2.221	0.835 - 3.519	0.148 - 6.460
group 10W	1.826 \pm 0.764	1.238 - 2.413	0.541 - 2.716
group DM	1.003 \pm 1.482	0.062 - 1.945	0.125 - 5.251
p-Akt/Akt			
group C	1.677 \pm 1.549	0.741 - 2.613	0.476 - 6.514
group 7W	1.194 \pm 0.619	0.820 - 1.568	0.480 - 2.547
group 10W	1.013 \pm 0.199	0.860 - 1.166	0.763 - 1.367
group DM	0.384 \pm 0.392	0.121 - 0.648	0.035 - 1.413
PECAM-1			
group C	1.690 \pm 0.965	1.107 - 2.273	0.446 - 3.550
group 7W	1.143 \pm 0.568	0.783 - 1.504	0.394 - 2.366
group 10W	1.018 \pm 0.458	0.666 - 1.370	0.568 - 1.973
group DM	0.102 \pm 0.087	0.047 - 0.157	0.026 - 0.335

eNOS, endothelial nitric oxide synthase; p-MYPT1, phosphorylated myosin phosphatase target subunit 1; PECAM-1, platelet endothelial cell adhesion molecule-1

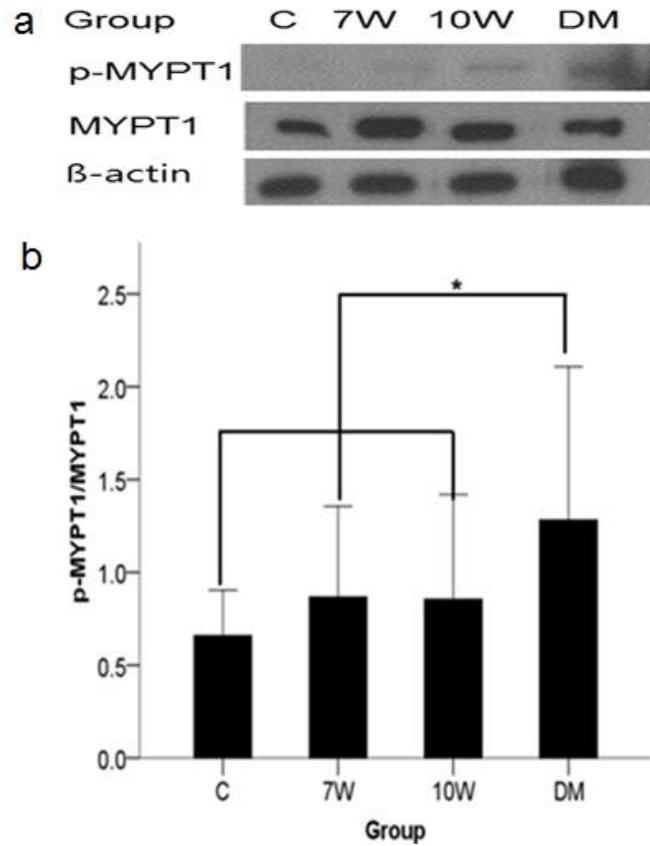


Figure 7. (a) Western blot analyses of total and phospho-MYPT1. (b) Ratio of phospho-MYPT1 to total MYPT1 protein expression. * $P < 0.05$. C, control; p-MYPT1, phosphorylated myosin phosphatase target subunit 1.

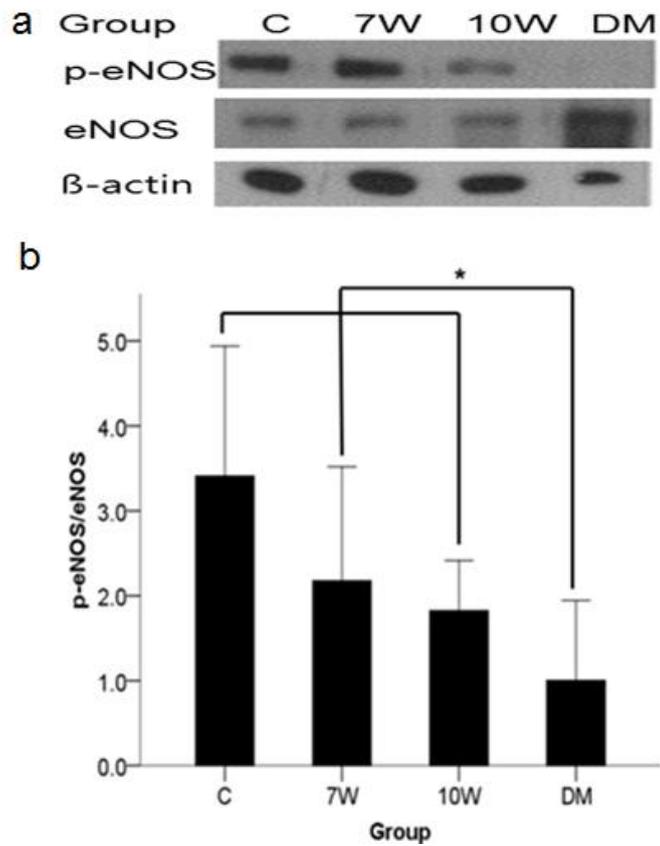


Figure 8. (a) Western blot analyses of total and phpspho-eNOS (b) Ratio of phospho-eNOS to total eNOS protein expression. * $P < 0.05$. C, control; eNOS, endothelial nitric oxide synthase.

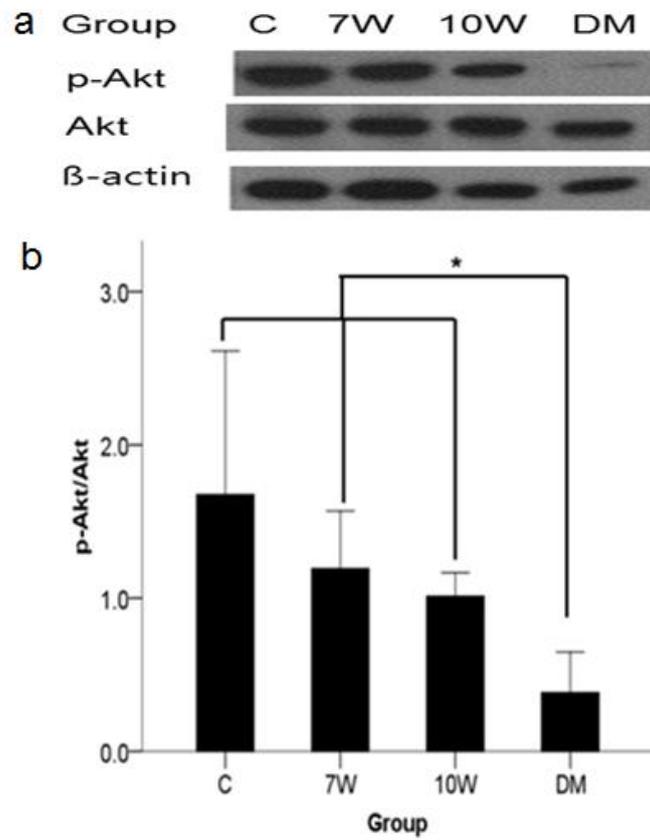


Figure 9. (a) Western blot analyses of total and phpspho-Akt (b) Ratio of phospho-Akt to total Akt protein expression. * $P < 0.05$. C, control.

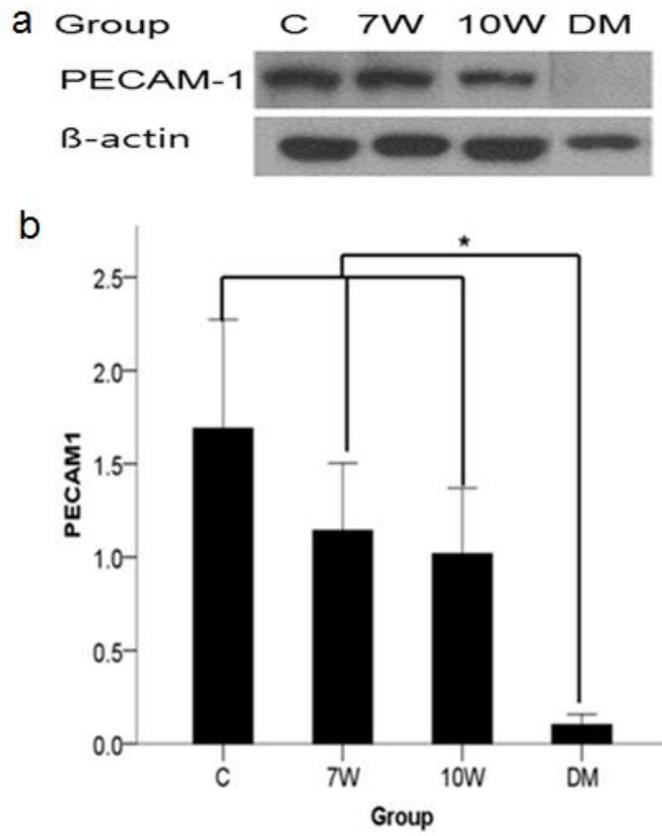


Figure 10. (a) Western blot analyses of PECAM-1 (b) Protein expression of PECAM-1. * $P < 0.05$. C, control; PECAM-1, platelet endothelial cell adhesion molecule-1.

DISCUSSION

Changes of erectile function were evaluated at different stages of streptozotocin-induced diabetic rats.² Cho and collaborators found that erectile function impairment depended on time since DM induction.² No evidence of impairment in the erectile function was observed until 6 weeks after DM induction. Erectile function started to deteriorate after 8 weeks of DM induction. ICP/MAPs obtained with low-frequency stimulation in 8-week diabetic rats were significantly lower than those in normal controls. In 10-week diabetic rats, both ICP/MAP and AUC/MAP significantly decreased compared with normal controls even at high-frequency stimulation. Based on another previous animal experiments, strict glycemic control is recommended to improve erectile function to near-normal status.³ After administration of daily multiple NPH injections for four weeks in 10-week diabetic rats, no differences in all erectile parameters were shown compared with normal controls.

The duration and severity of ED have been associated with the duration of DM and the glycemic control. A higher incidence of ED was reported in men with DM compared to those without DM in the Massachusetts Male Aging Study.²⁴ Johannes and collaborators estimated that ED was present with in approximately 10 years after diagnosis of DM. From the Health Professional Follow-Up Study cohort, Bacon and collaborators reported that the risk of ED was increasing positively associated with the increasing duration of DM, especially for men diagnosed more than 20 years previously.²⁵ In addition, Fedele and collaborators, in a cross-sectional study, reported that the odds ratios for ED were 1.3 and 2.0 in patients with DM lasting 6–10 and 11–30 years, respectively, in comparison with those with DM lasting less than 5 years.²⁶ Also, the odds ratios for ED were 1.7 and 2.3 in men with fair and poor control, respectively, for men

with good metabolic control. The purpose of this study was to evaluate the effects of glycemic control start time on DMED in diabetic rat model.

The benefits of early insulin treatment on the course of diabetic complications have been reported since 30 years ago.²⁷⁻²⁹ But in vivo studies were based on cellular dysfunction and did not consider metabolic and structural changes. Additionally, comparative results of compromised erectile function in DMED have been inadequately investigated. Cellek and collaborators concluded that neuronal apoptosis occurring in later-phase diabetic neuropathy was irreversible, despite insulin replacement.²¹ However, no study has directly compared different treatment timings after inducing DM. Instead, many studies assessed recovery of erectile function after DMED, using various start times for treatment after DM induction. Park and collaborators reported that conventional insulin treatment (daily single injection of NPH) was ineffective for restoring erectile responses 10 weeks after DM induction.³⁰ Yang and collaborators studied 4-week treatment with daily NPH injections, 8 weeks following DM induction.³¹ They observed increased erectile response in diabetic rats after insulin treatment, but no statistically significant difference compared with untreated diabetic rats ($P > 0.05$). Wang and collaborators started insulin treatments 8 weeks after inducing DM.³² After 6 weeks of treatment, the insulin monotherapy group partially improved compared with untreated diabetic rats.

In this study, the start time of insulin treatment was determined based on results from previous experiments.² Considering the results, we selected 7 weeks after DM induction as early treatment, and 10 weeks as timing when overt DMED occurs. And the level of glycemic control was determined using a clinically practical approach. Insulin treatment has an important position in the management of diabetes.³³ In addition, glycemic control has an independent, inverse correlation with DMED.³⁴ Moreover, based on previous animal experiments, strict glycemic control is

recommended to improve erectile function to near-normal status.³ However, intensive glycemic control is difficult for patients to maintain and can cause hypoglycemia.³⁵ Thus, to reduce the risk of severe hypoglycemia, clinical guidelines for DM treatment have proposed maintaining glucose levels slightly higher than those needed for intensive control.³⁶⁻³⁸ Similar to a previous study, glucose levels were controlled using a daily single injection of NPH.³

Although insulin has been the mainstay for the treatment of uncontrolled DM, maintaining tight glycemic control without adverse consequences such as hypoglycemia and weight gain still remains a challenge.^{33,39} Adipose tissue plays a dynamic role in body energy homeostasis by acting as an endocrine organ. In addition to the energy homeostasis, the adiposity hormone leptin also plays an important role in glucose metabolism.⁴⁰ Yu and collaborators demonstrated that leptin had glucose lowering effects in diabetic rat model.⁴¹ Insulin is needed for the synthesis and storage of triacylglycerol into adipose tissue, so body fat is depleted without insulin therapy in DM. This progressive loss of body fat is followed by decrease of plasma leptin levels.⁴² Insulin resistance is known to be closely associated with sex hormone concentration.⁴³ Testosterone levels are lower in DM patients because of decreased levels of sex-hormone binding globulin. However, an increase in leptin levels with weight gain significantly decreased the production of testosterone from Leydig cells.⁴⁴ So, the relationship between androgens, adipose tissue, and insulin sensitivity has not been clearly verified yet.

This study evaluated the effects of different glycemic-control start times on erectile function in diabetic rats. Group DM had significantly weaker responses than control group C. All treated groups showed significant recovery in erectile function compared with group DM, and ICP/MAP ratio gradually increased with earlier glycemic control start times. Even so, the early treatment group, which was expected to have uncompromised erectile

function, did not recover to normal control group levels. Evaluating structural changes, % α -SMA significantly decreased in group DM, and groups 7W and 10W had similar % α -SMA as group C. Compared with group C, apoptotic cell density significantly increased in DM groups, except for early treatment group 7W. Molecular changes showed similar results in the treatment groups compared with group C. In comparison with group 10W, group 7W appeared to have erectile function more close to that of normal controls, but the differences were not statistically significant. Therefore, the degree of glycemic control can be considered to be more important than the start timing of glycemic control for preventing or treating DMED.

In our study, recovery of erectile function tended to increase as treatment start time became earlier; however, even early treatment group did not recover erectile function to the level of normal controls. Group 10W's increase in erectile response resembled the once-daily insulin injection group in a previous study.³ However, after stimulation with 10 Hz and 20 Hz frequencies, the ratios of ICP/MAP and AUC/MAP were higher in group 7W than in group 10W. Considering that each group comprised only 15 rats, we believe the differences between groups 7W and 10W could have reached significance in experiments with larger groups.

We estimate that degree of glycemic control exerts a greater effect on erectile function recovery than does treatment start time. However, considering the difficulties of maintaining strict glycemic control in real practice, the effects of early glycemic control may still be important for DMED recovery. Although apoptotic cell density significantly increased in group 10W, α -SMA expression and cavernous endothelial content did not significantly decrease. Furthermore, Western blot analysis showed no significant differences in NO pathways among group C, 7W, and 10W. These results suggest that the apoptotic response accelerates 7-10 weeks after inducing DM. Jin and collaborators found that apoptotic cells were not

limited to cavernous endothelial and smooth muscle cells but were detected in other cavernous areas.³⁹ Therefore, in contrast with apoptotic response, structural changes explained by α -SMA expression and PECAM-1 were able to be restored until group 10W. This recovery resembled the results of protein Western blot analyses.

There were several limitations of this study. First, the degree of glycemic control could not reflect actual human conditions. However, glucose levels in the two treatment groups resembled those receiving once-daily injections in previous studies; thus, glycemic control was maintained at a similar level across studies. Second, the treatment period for group 10W was only 4 weeks, which may be considered too short to accurately evaluate the effects of glycemic control. However, erectile function of group 7W also did not approach up to group C. And DM was induced more severely here than in previous studies, so it was difficult to keep the untreated rats alive past the expected schedule. Third, we did not investigate all pathophysiological mechanisms associated with restoring DMED. We excluded analysis of neuronal NOS, which was included in previous studies, because the role of this molecule in restoring erectile function was unclear. Instead, we added PECAM-1 to Western blot analysis to assess endothelial changes.

CONCLUSIONS

In conclusion, erectile function of diabetic rats recovered closer to levels seen in normal controls if DM treatment began earlier, but did not reach that of normal controls. We can expect that degree of glyceimic control was more important than timing of DM treatment start. Considering that early treatment group 7W did not recover erectile function up to normal group C, additional experiments will help compare treatment effects between degree of glyceimic control and start time of glyceimic control.

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국문 초록

Streptozotocin 유도 당뇨병 백서에서 혈당 조절의 시작 시기가 발기력에 미치는 영향

서론: 당뇨병 남성에서 발기부전이 나타날 위험성은 당뇨병의 유병기간 및 혈당 조절의 정도와 밀접한 관련성을 지니고 있다. 본 연구에서는 당뇨병 유발 백서모델에서 혈당 조절 시작 시기의 차이에 따른 발기력의 변화와 이에 관여하는 병태생리기전을 알아보려고 하였다.

방법: 8주령 수컷 백서 60마리를 정상 대조군과 streptozotocin으로 당뇨병 유발 후 비치료군, 7주, 10주 경과 후 인슐린 투여 시작 군 (각 군 n=15)으로 구분하였다. 당뇨병 유발 백서는 1일 1회 인슐린 (NPH 3-6 단위) 투여로 혈당을 조절하였으며, 혈당 조절의 수준은 엄격하지는 않았다. 당뇨병 유발 14주 후 모든 백서에서 해면체신경 전기자극 (10 Hz and 20 Hz under 3 V, 0.2 msec for 30 sec)을 시행하여 평균 동맥압에 대한 최대 해면체내압의 비 (ICP/MAP)를 구하였다. 또한 음경조직을 이용하여 TUNEL 염색을 시행하고, 해면체 평활근에 대한 면역조직화학검사를 시행하였다. 그리고, 단백을 추출하여 Western blot으로 eNOS, Akt, MYPT1, PECAM-1 등의 단백질 발현을 조사하였다.

결과 : 인슐린 치료군에 비해 비치료 당뇨병군에서 ICP/MAP 값이 현저하게 저하되었다 (10주 vs DM, $p < 0.001$). 당뇨병군에서 혈당 조절의 시작 시기가 빠를수록 ICP/MAP 값은 유의하게 증가하였으나 대조군 수준으로 회복하지는 못하였다 (정상 vs 7주, $p < 0.001$). 당뇨병 유발로 유의하게 감소된 해면체 평활근의 함량도 혈당 조절의 시작 시기가 빠를수록 증가하였으며, 혈당 조절을 시행한 두 군 (7주와 10주)에서는 정상 대조군과 차이를 보이지 않았다. TUNEL 염색으로 측정된 세포고사지수는 혈당 조절을 조기에 시작한 군 (7주)에서만 비치료 당뇨병군과 차이를 보였다. 해면체 조직에서 추출한 단백질들은 모든 종류에서 당뇨병 치료 시기가

빠를수록 정상 대조군에 가까워지는 경향을 보였다 (eNOS, Akt, MYPT1, and PECAM-1).

결론: 당뇨병 백서모델에서 혈당 조절을 조기에 시행할수록 발기력이 호전되었으나 정상 수준으로 회복하지는 못하였다. 이는 당뇨병성 발기부전의 예방과 치료에서 혈당 조절의 시작 시기가 중요하나 혈당 관리의 정도가 더욱 중요함을 시사한다.

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주요어 : 발기부전, 당뇨, 당뇨병 백서 모델

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