

# The impact of liraglutide treatment on erectile function of the diabetic rats

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## ABSTRACT

**Objectives:** Glucagon like peptide-1 (GLP-1) is a hormone released from intestinal L-cells following nutrient consumption. It potentiates secretion of insulin from pancreatic beta-cells thus GLP-1 analogues are used for the treatment of type-2 diabetes mellitus (T2DM). This study aims to evaluate impact of GLP-1 receptor agonist liraglutide on erectile function of diabetic rats.

**Methods:** Male Sprague-Dawley rats (n = 30, 13-weeks old, 240-335 gr) were fed with fatty diet for 2-weeks and divided into 3 groups (n = 10 each). The rats in the first group served as controls (Group C) whereas the rats in the remaining two groups were injected with streptozocin and became T2DM for forming diabetic group (Group D) and treatment group (Group DT). Rats in group D received citrate buffer injections whereas rats in the group DT received liraglutide injections (0.3 mg/kg/12h) subcutaneously. Erectile functions of all rats were evaluated with intracavernosal pressure (ICP)/mean arterial pressure (MAP) measurements. Moreover, plasma sex hormone levels (Testosterone, FSH, LH) were measured and histological assessment of midpenile tissue were performed (Collagen-Type-IV, rat epithelial antigen-1, nNOS).

**Results:** Maximum ICP/MAP ratios were  $0.790 \pm 0.164$ ,  $0.263 \pm 0.139$  and  $0.652 \pm 0.131$  in Group C, Group D and Group DT. Although mean ICP/MAP ratios were similar in Group C and Group DT ( $p = 0.076$ ), mean ICP/MAP ratio was significantly lower in Group D ( $p < 0.001$ ). Testosterone and FSH results were significantly lower in the Group D as well ( $p = 0.001$ ). Histological analyses revealed that nNOS ( $p < 0.001$ ), rat epithelial antigen-1 ( $p = 0.016$ ) and muscle/collagen ratio ( $p = 0.015$ ) were also lower in Group D, compared with the other groups.

**Conclusions:** GLP-1 receptor agonist liraglutide demonstrated protective effects on the erectile tissues of the diabetic rats. Clinical trials are required to confirm if liraglutide treatment has similar beneficial effects on men who have T2DM.

**Keywords:** Erectile dysfunction, GLP-1 agonist, intracavernosal pressure, liraglutide, nNOS, type 2 diabetic rat

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**E**rectile dysfunction (ED) is a permanent impairment in initiating and/or sustaining sufficient erection for a satisfactory sexual performance [1]. It is estimated that there are 18 million ED patients in United States of America [2]. The most important risk factors for ED are ageing, atherosclerosis, diabetes mellitus (DM), hypertension, smoking and dyslipidemia [3, 4]. ED frequency in DM patients ranges between 35-90% [5, 6]. Vascular, endothelial, neuronal, endocrine and metabolic pathways play role in the pathogenesis of ED in DM patients [7-12]. In diabetic patients, ED occurs earlier than in the normal population and these cases respond less to phosphodiesterase type 5 inhibitors, which is considered as the first line treatment of ED [13].

Liraglutide is a long acting glucagon-like peptide-1 (GLP-1) receptor agonist used in the treatment of DM [14]. This molecule enhances insulin secretion by stimulating beta cells of the pancreas. Liraglutide controls blood glucose level, while at the same time delays gastric discharge and suppresses postprandial glucagon secretion [15]. Several studies have proven that GLP-1 receptors are also present in extrapancreatic tissues [16, 17]. Although the effects of other new generation DM drugs on erectile function have been examined [18-20], the effect of GLP-1 receptor agonists on diabetic ED has not been analyzed. Although Galli *et al.* [21] have received a patent on that the GLP-1 receptor agonists can be beneficial for the ED treatment by activating GLP-1 receptors in the penile tissue, there are not any clinical or translational data to confirm this hypothesis. Yue *et al.* [22] and Yuan *et al.* [23] have suggested that GLP-1 receptor agonists may protect the endothelial function, regulation of smooth muscle dysfunction, oxidative stress and autophagy, independently of a glucose-lowering effect and regulating oxidative stress, by acting through the Akt/eNOS signal pathway and the RhoA/ROCK pathway but there is not yet enough scientific data on this topic.

Therefore, we aimed to reveal the hormonal, functional and morphological effects of liraglutide treatment on erectile function of rats with type 2 DM (T2DM).

## METHODS

This study was carried out at Istanbul Bagcilar Training and Research Hospital Experimental Research and Skill Development Training Centre (BADABEM) with the approval of project numbered 2016-30 from Bagcilar Training and Research Hospital Local Ethics Committee of Animal Tests (SBU B.E.H HADYEK). All expenses of the study were met from the research grant provided by Istanbul Bagcilar Training and Research Hospital Training Planning Committee.

A total of 30 healthy male Sprague-Dawley rats weighing between 240-335 g were included in the study. During the study, rats were fed with tap water (*ad libitum*) with 2% cholesterol and 10% fat containing rat feed (Ziegler Bros. Gardners, PA). The animals were monitored at a temperature of 22°C for 12 hours in the light and 12 hours in the dark. After feeding them in physiological cages with a cycle of 12-hour night/day for 2 weeks, 10 rats were randomly assigned to form the Control Group (Group C). The remaining 20 rats received intraperitoneal streptozocin (STZ) injection (30 mg/kg) for 2 times within a period of 3 days [24]. Blood glucose levels were measured daily and then weekly throughout the period of the study from the rats' tails with the glucometer (Accu-Chek, Roche, Mannheim, Germany) until it is confirmed that the rats are hyperglycemic (blood glucose level > 300 mg/dL). For measurement of the sensitivity to a challenge of insulin, 1 IU/kg bovine insulin in phosphate-buffered solution (PBS) (1 IU/mL) was administered by intraperitoneal injection and tail snip capillary blood samples were collected at 0, 15, 30, 45, 60, 90, and 120 minutes after injection for obtaining a blood glucose response curve.

After these 20 rats became hyperglycemic, they were randomly divided into two groups. Rats assigned to the Diabetic Treatment Group (Group DT) were injected subcutaneously with liraglutide (Victoza®, Novo Nordisk, Denmark) for 12 weeks (0.3 mg/kg/12h) whereas rats in the Diabetic Group (Group D) were given citrate buffer (0.25 ml/kg/12h) solution for 12 weeks.

Liraglutide dosage has been used with reference

to previous studies [17, 25]. After 12 weeks of treatment, rats were anaesthetized with ketamine (60 mg/kg) and xylazine (7.5 mg/kg) intraperitoneally for the physiological testings (24 hours after the last injection to Group D and Group DT). A single dose of ketamine (50 mg/kg) was repeated as needed to continue spontaneous breathing of the rats during the measurements.

### Intracavernosal Pressure / Mean Arterial Pressure Measurements

After the anesthesia, the rats were placed in supine position and the incision site was cleared. With transverse neck incision, the left internal carotid artery was identified next to the trachea. The artery was ligated distally and cannulated with PE50 tube after a small incision. PE50 tube was connected to booster unit with pressure transducer (Commat Pharmacology & Physiology Instruments, Ankara, Turkey). This booster unit was connected to the information conversion module (MP35 data acquisition system, Ankara, Turkey) so that the systemic Mean Arterial Pressure (MAP) could be recorded and measured from the computer (Biopac Systems Inc, CA, USA).

Afterwards, a circular incision was performed onto the penis and a 24 G needle tip was placed into the right crurale of the penis for the measurement of Intra Cavernosal Pressure (ICP) (mmHg). Subsequently, a midline laparotomy incision was performed and the main pelvic ganglion was identified with 3.5 × operation magnifying glass in the dorsal section of the prostate. The cavernosal nerve was detected from the satellite ganglia in the lateral of the main pelvic ganglion. The nerve was captured with bipolar electrode just at the distal part of the main ganglion. The electrode line was connected to the STPT02 stimulator (COMMAT Pharmacology & Physiology Instruments, Ankara, Turkey) and the stimulation parameters were adjusted as; 1.5 milliamps, 20 Hz, 5 milliseconds pulse interval, 35 milliseconds delayed, 7.5 volts for 60 seconds. During the nerve stimulation, the maximum ICP/MAP was calculated and the percent of these values in the confidence interval of 95% was reported. High-dose single stimulation was used due to the surgeon and technical equipment performing the procedure, and either single stimulation or low dose to high dose stimulation is not standard. In this study, ICP

measurement was made in accordance with the measurement made by Mullerad *et al.* [26].

### Serum Analyses

The intracardiac blood samples were placed in biochemical tubes (BD Vacutainer SST II, Beliver Industrial Estate, UK) and centrifuged at 20,000 rpm for 90 seconds for the decomposition of the serum. The FSH, LH and total testosterone levels were measured in a biochemical analyzer (Roche Cobas 6000 Immunoassay, Roche Diagnostics, Basel Switzerland) according to the manufacturer's instructions by using the sandwich enzyme immunoassay method.

### Histopathological Examinations

All penile tissue specimens were first fixated in a 10% Neutral Buffer formaldehyde solution for light and immunofluorescence microscopic examination. After the fixation process, the tissue samples were placed in trays and washed under running water for 2 hours. For draining the water, the tissues were passed from the alcohol series (70%, 80%, 90%, 100%) at increasing degrees. Subsequently, the tissues were passed from xylol solution and then buried into the molten paraffin. Both Hematoxylin-Eosin Staining and Immunohistochemical Staining for rat epithelial antigen-1 (RECA-1 Antibody, ab9774, Abcam, Cambridge, UK), Collagen IV (Collagen IV Antibody, ab6586, Abcam, Cambridge, UK) and neuronal nitric oxide synthase (nNOS Antibody, ab5586, Abcam, Cambridge, UK) were performed. The sections were evaluated in the Leica DC-4000 (Germany) computer-supported imaging system in the Leica Q Vin 3 program. During polarized light microscopy and immunofluorescence microscopic examinations nNOS and RECA-1 expressions could be evaluated whereas we could not obtain sufficient staining for Collagen Type IV. Therefore, the evaluation of collagen accumulation was carried out by polarized light microscopy with Masson's Trichrome staining (Table 1).

### Statistical Analysis

The statistical analyses were performed by using the NCSS (Number Cruncher Statistical System) 2007 Statistical Software (Utah, USA) package program. Besides the descriptive statistical methods (mean,

**Table 1. Histopathologic scoring table for nNOS, RECA-1, collagen type IV expressions 0: No staining; 1: Minimal level of positivity (< 10%); 2: Medium level of positivity (< 10%-50%); 3: High level of positivity (> 50%)**

<b>Histopathological scoring table</b>			
<b>N-NOS</b>	<b>Group C</b>	<b>Group D</b>	<b>Group DT</b>
1	3	1	2
2	2	1	2
3	2	2	2
4	2	1	2
5	2	1	2
6	3	2	3
7	2	1	2
8	3	1	3
9	2	1	
10	3	1	
<b>RECA-1</b>	<b>Group C</b>	<b>Group D</b>	<b>Group DT</b>
1	1	0	0
2	1	1	2
3	2	1	0
4	2	1	1
5	2	1	1
6	1	0	1
7	1	1	0
8	2	0	1
9	2	0	
10	2	1	
<b>Collagen type IV</b>	<b>Group C</b>	<b>Group D</b>	<b>Group DT</b>
1	No staining	No staining	No staining
2	No staining	No staining	2
3	1	2	2
4	1	No staining	2
5	No staining	1	No staining
6	No staining	No staining	No staining
7	No staining	No staining	No staining
8	No staining	No staining	No staining
9	1	2	
10	No staining	2	
<b>Collagen review with trichrome</b>	<b>Group C</b>	<b>Group D</b>	<b>Group DT</b>
1	1	3	1
2	1	2	1
3	2	3	1
4	1	1	2
5	1	3	1
6	1	3	1
7	1	3	2
8	1	3	1
9	1	2	
10	2	3	

standard deviation, median and interquartile range), Kruskal Wallis test was used for comparing the variables with non-normal distribution between the groups, Dunn's multiple comparison test was used for subgroup comparisons, and Chi-square test was used for qualitative data comparisons. The results were evaluated at  $p < 0.05$  significance level

## RESULTS

Twenty-four hours after the initiation of the treatment, 2 rats died in Group DT and the study was completed with 28 rats, 12 weeks after the injection of STZ (Group DT: 8, Group D: 10, Group C: 10 rats). There was no statistically significant difference among the weight averages ( $p = 0.448$ ) and mean blood glucose levels ( $p = 0.546$ ) of the rats in different groups before the treatment. However, rats in Group D were significantly lighter ( $p = 0.001$ ) and their blood glucose levels were significantly higher ( $p = 0.001$ ) compared with the rats in Group C and Group DT (Fig. 1).

### Intracavernosal Pressure / Mean Arterial Pressure Measurements

No statistically significant difference was observed between the MAP values of Group C, Group D and Group DT ( $p = 0.363$ ). Maximum ICP/MAP rates were  $0.790 \pm 0.164$ ,  $0.263 \pm 0.139$  and  $0.652 \pm 0.131$  in Group C, Group D and Group DT respectively. Although there was no statistically significant difference

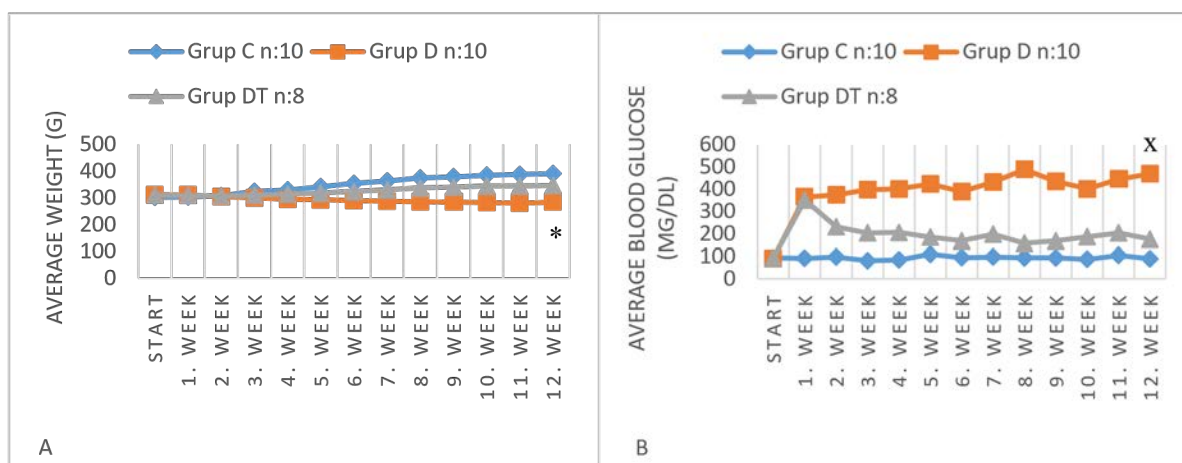
between Group C and Group DT ( $p = 0.076$ ), the maximum ICP/MAP values of Group D were significantly lower than Group C ( $p = 0.001$ ) and Group DT ( $p = 0.001$ ) (Table 2 and Fig. 2).

### Serum Analyses

Average total testosterone values are shown in Table 3. Mean total testosterone level was significantly lower in Group D compared with Group C ( $p = 0.001$ ) and Group DT ( $p = 0.001$ ). Mean total testosterone level of Group DT was also lower than that of Group C but this difference did not reach to a statistically significant level ( $p = 0.076$ ) (Table-3). FSH values were also significantly lower in Group D compared with Group C ( $p = 0.001$ ). Although mean FSH in Group C was lower than Group DT, this was not statistically significant ( $p = 0.076$ ). LH values of Group C were lower than Group D ( $p = 0.001$ ) and Group DT ( $p = 0.001$ ). Although the mean LH levels in Group DT were greater than Group D, this was not statistically significant ( $p = 0.086$ ) (Table 3).

### Histopathological Examinations

Statistically significant difference was observed between the nNOS values of Group C, Group D and Group DT ( $p = 0.0001$ ). High level of positivity in Group D was found lower than the Group C and Group DT (Table 4, Fig. 3). Statistically significant difference was also observed between the RECA-1 values of Group C, Group D and Group DT ( $p = 0.016$ ). Medium level of positivity in Group D was



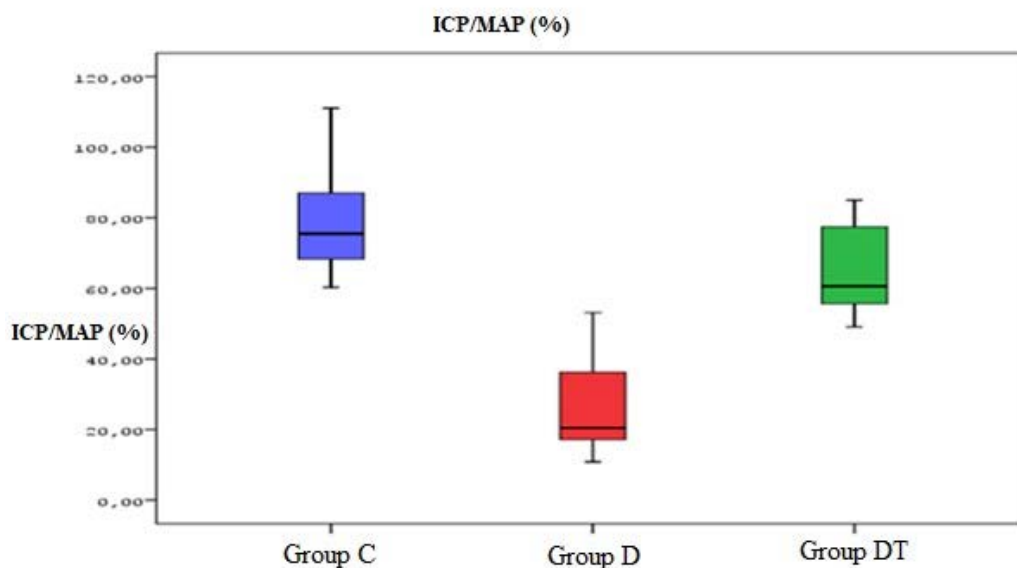
**Fig. 1.** (A) Weekly weight measurement average results ( $*p = 0.001$  for Group D), (B) Weekly blood glucose measurement average results ( $xp = 0.001$  for Group D)

**Table 2. Intracavernosal pressure (ICP) and mean arterial pressure (MAP) measurements, ICP/MAP ratios and intergroup evaluation results**

Kruskal Wallis Test		Group C	Group D	Group DT	p value
<b>ICP max</b>	Mean ± SD	32.06 ± 22.29	9.33 ± 7.23	33.35 ± 7.06	<b>0.001</b>
	Median (IQR)	31.9 (12.51-44.15)	7.1 (5.06-10,43)	34.7 (29.05-38.8)	
<b>MAP ave</b>	Mean ± SD	38.75 ± 23.18	37.41 ± 17.36	51.57 ± 9.52	<b>0.363</b>
	Median (IQR)	38.42 (16.41-58.34)	37.56 (22.68-53.5)	47.5 (45.38-62.78)	
<b>ICP/MAP</b>	Mean ± SD	0.790 ± 0.164	0.263 ± 0.139	0.652 ± 0.131	<b>0.0001</b>
	Median (IQR)	0.755 (0.665-0.9)	0.205 (0.16-0.378)	0.606 (0.551-0.782)	
<b>ICP/MAP (%)</b>	Mean ± SD	79.13 ± 16.26	26.3 ± 13.91	65.18 ± 13.14	<b>0.0001</b>
	Median (IQR)	75.5 (66.88-90)	20.45 (16.03-37.93)	60.6 (55.08-78.2)	
<b>Dunn's multiple comparison test</b>		<b>ICP/MAP (p value)</b>		<b>ICP/MAP(%) (p value)</b>	
Group C / Group D		<b>0.0001</b>		<b>0.0001</b>	
Group C / Group DT		0.076		0.076	
Group D / Group DT		<b>0.001</b>		<b>0.001</b>	

found lower than the Group C and Group DT (Table 4 and Fig. 4). The collagen distributions of Group C, Group D and Group DT were also significantly differ-

ent ( $p = 0.015$ ). High level of positivity in Group D was found higher than the Group C and Group DT (Table 4 and Fig. 3).



**Fig. 2.** Intracavernosal pressure (ICP) / mean arterial pressure (MAP) ratios, Group C:  $0.79 \pm 0.16$ ; Group D:  $0.26 \pm 0.13$ , Group DT:  $0.65 \pm 0.13$ .

**Table 3. Serum mean total testosterone(ng/ml), FSH(IU/l), LH(IU/l) values and intragroup evaluation results**

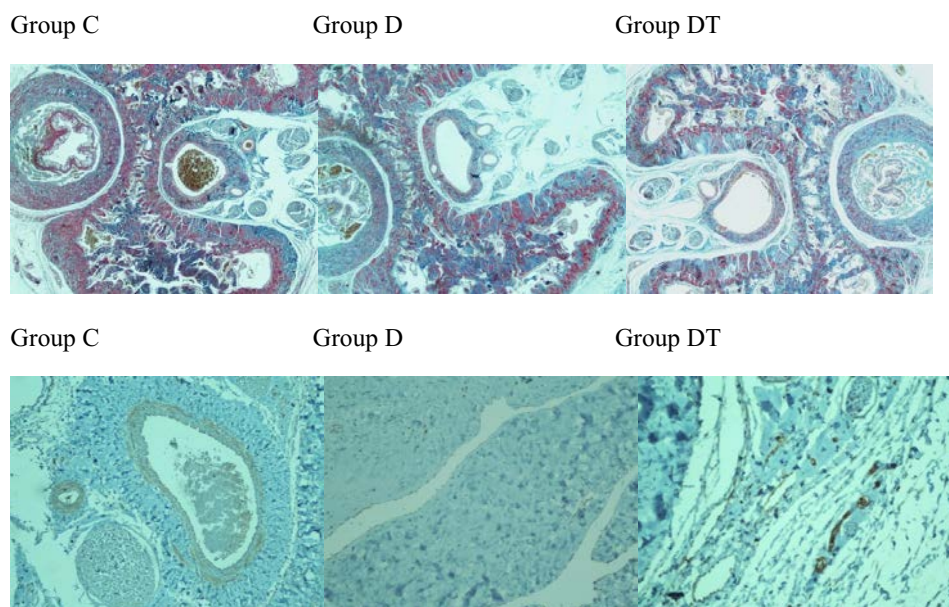
Kruskal Wallis Test		Group C	Group D	Group DT	p value
<b>Total testosterone (ng/mL)</b>	Mean ± SD	2.57 ± 0.63	0.58 ± 0.21	1.61 ± 0.23	<b>0.0001</b>
	Median (IQR)	2.58 (1.97-3.0)	0.6 (0.525-0.671)	1.63 (1.43-1.79)	
<b>FSH (IU/L)</b>	Mean ± SD	0.107 ± 0.006	0.054 ± 0.007	0.132 ± 0.01	<b>0.0001</b>
	Median (IQR)	0.107 (0.102-0.11)	0.052 (0.048-0.06)	0.130 (0.128-0.139)	
<b>LH (IU/L)</b>	Mean ± SD	0.335 ± 0.009	0.398 ± 0.028	0.399 ± 0.027	<b>0.0001</b>
	Median (IQR)	0.335 (0.33-0.339)	0.397 (0.378-0.416)	0.402 (0.368-0.421)	
Dunn's Multiple Comparison Test		Total Testosterone	FSH	LH	
Group C / Group D		<b>0.0001</b>	<b>0.0001</b>	<b>0.0001</b>	
Group C / Group DT		0.076	0.076	<b>0.0001</b>	
Group D / Group DT		<b>0.001</b>	<b>0.001</b>	0.859	

**DISCUSSION**

The pathophysiology of diabetes related ED has not been fully enlightened, however increased hyperglycemia-induced glycosylation and reduced NOS phosphorylation are thought to be the key mechanisms [27]. Albersen *et al.* demonstrated decreases in nNOS expression, impairment in endothelial integrity, and

decreases in smooth muscle/collagen ratio in cavernosal tissues of the diabetic rats, supporting these theories [24].

Better glycemic control is associated with improved diabetes-related complications. Among the new generation DM treatments, pioglitazone has been found to be associated with improved erectile functions after radical prostatectomy [18, 20], which has



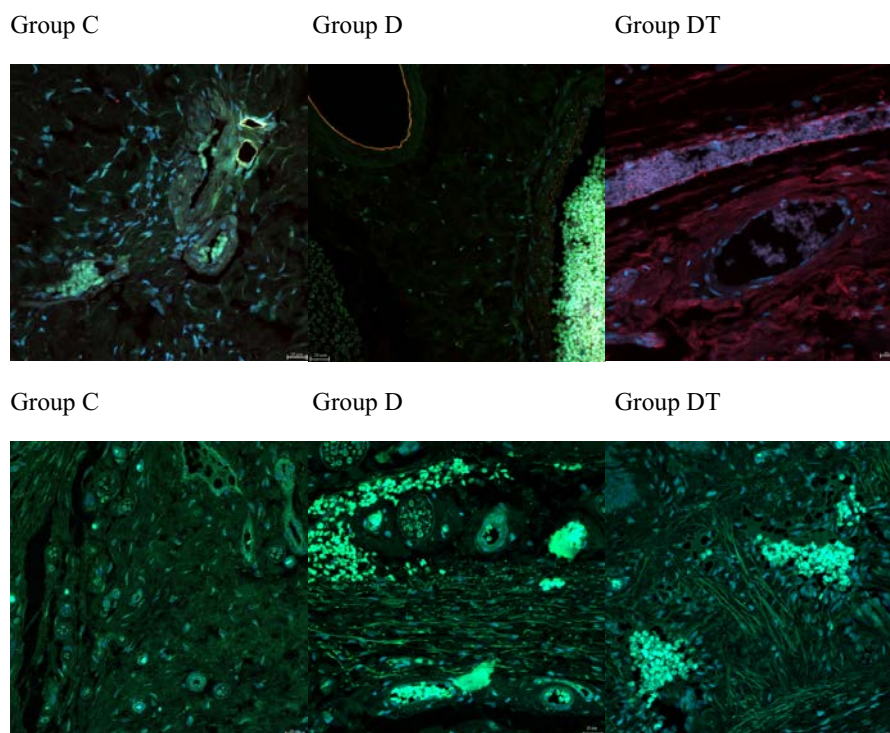
**Fig. 3. First row: Collagen tissue in stroma and vein wall in Grup C, increase in Group D, minimal increase in Group DT. Second row: Positivity in vein wall and nerve tissue in Group C with nNOS (× 10), No positivity in Group D with nNOS (× 20), Positivity in vein wall and nerve tissue in Group DT with nNOS (× 10).**

**Table 4.** Histopathologic scoring table and intragroup evaluation results for nNOS, RECA-1, collagen type IV expressions

Chi-Square Test		Group C		Group D		Group DT		p value
		n	%	n	%	n	%	
N-NOS	Positive at minimal level	0	0.00	8	80.00	0	0.00	<b>0.0001</b>
	Positive at medium level	6	60.00	2	20.00	6	75.00	
	Positive at high level	4	40.00	0	0.00	2	25.00	
RECA-1	Negative	0	0.00	4	40.00	3	37.50	<b>0.016</b>
	Positive at minimal level	4	40.00	6	60.00	4	50.00	
	Positive at medium level	6	60.00	0	0.00	1	12.50	
Trichrome-Collagen	Positive at minimal level	8	80.00	1	10.00	6	75.00	<b>0.015</b>
	Positive at medium level	2	20.00	2	20.00	2	25.00	
	Positive at high level	0	0.00	7	80.00	0	0.00	

been explained with the presence of its extrapancreatic receptors. Similarly, GLP-1 receptors are also detected in extrapancreatic tissues such as the myocardial and pulmonary arterial systems, which exert their effects via NO dependent mechanisms (possibly NOS2 activation over  $G_{i\alpha}$  and MAP kinase dependent p38) [16, 17]. Zhou *et al.* Have shown that GLP-1 agonist liraglu-

tide treatment protects eNOS activity with inhibition of NF- $\kappa$ B pathway, in addition to the maintaining glycemic control in diabetic rats and has direct beneficial effects against diabetic nephropathy [17]. GLP-1 receptors also exist in the penile tissues and GLP-1 receptor agonists are hypothesized to be beneficial in the ED treatment [21]. In a Chinese study Yue *et al.*



**Fig. 4.** First row: Fluorescence microscopic image of nNOS positivity in vein wall and nerve tissue. Second row: Fluorescence microscopic image of endothelial RECA-1 expressions.



[22] demonstrated that GLP-1 receptor agonist liraglutide can protect the endothelial functions of diabetic rats by acting through the Akt/eNOS signal pathway. However, the authors did not conduct any physiological testings for the assessment of erectile function of these rats. Yuan *et al.* [23] conducted physiological testings for the assessment of erectile function but they conducted experiments in type 1 diabetes model, which impeded generalization to the general diabetic population. In a retrospective study conducted in 2015 shown that adding GLP-1 agonist, life style change, metformin and testosterone replacement therapy improved erectile dysfunction due to hypogonadism in patients with T2DM [28].

In order to elucidate the actual impact of GLP-1 receptor agonists on erectile function of T2DM rats, we have conducted hormonal, functional and morphological analyses on rats with T2DM. Our results revealed more favorable ICP/MAP values in diabetic rats treated with liraglutide compared to untreated diabetic rats. Moreover, we evaluated the endothelial function of diabetic rats after liraglutide treatment by comparing the morphologic changes in cavernous endothelium along with the RECA-1 expressions in corpus cavernosum. We found that the endothelial integrity was impaired in the diabetic rats and RECA-1 expressions decreased significantly, which could be protected with liraglutide treatment. Moreover, liraglutide treatment maintained the nNOS expressions, which was decreased in the diabetic rats.

Poor glycemic control is associated with decrease in cavernosal smooth muscle content, increase in the collagen amount and decrease in the contractile capacity of the penile tissues [19, 29, 30]. Although we aimed to evaluate the collagen type IV accumulation, which is known to play a significant role in the contractile capacity of cavernous tissue [31, 32], we could not achieve comparable staining. However, trichrome staining method revealed that smooth muscle/collagen ratio decreased in the diabetic rats, but it was maintained in the liraglutide treatment receiving rats. Future studies must elucidate the actual mechanisms explaining how GLP1 receptors ameliorate the detrimental effects of diabetes on the penile tissues.

DM also impairs erectile functions through their effects on the hypothalamohypophyseal axis. DM causes secondary testicular failure by decreasing gonadotropin hormone levels [33]. We also detected de-

creased total testosterone and FSH levels in the diabetic rat group whereas the levels of these hormones were preserved in the treatment group. There is a need for additional studies to understand the effects of diabetes and GLP-1 agonists on the hypothalamohypophyseal axis and testes.

To our knowledge, our study is the first study that evaluated the effects of GLP-1 agonists on erectile function in diabetic rats, both functionally and histologically. Our results revealed that liraglutide treatment has the potential of preserving the penile tissues and maintaining erectile functions in patients with DM. This study was done in rats, and it is much too early to extrapolate it to humans. Future clinical trials are required to confirm this hypothesis.

Limited histopathological examination and limited quantitative evaluation are among the limitations of the study.

## CONCLUSION

GLP-1 agonist liraglutide treatment has protective effects on erectile function of rats with T2DM. There is a need for clinical trials to verify whether liraglutide treatment has similar beneficial effects in patients with DM.

### Authors' Contribution

Study Conception: SG, MT, MGÇ, SS, YB; Study Design: SG, MT, MGÇ, SS, YB; Supervision: SG, MT, MGÇ, SS, YB; Funding: SG, MT, MGÇ, SS, YB; Materials: SG, MT, MGÇ, SS, HHT, YB, ECS, AS; Data Collection and/or Processing: SG, MT, MGÇ, SS, HHT, YB, ECS, AS; Statistical Analysis and/or Data Interpretation: SG, MT, MGÇ, SS, HHT, YB, ECS, AS; Literature Review: SG, MT, MGÇ, SS, HHT, YB, ECS, AS; Manuscript Preparation: SG, MT, MGÇ, SS, HHT, YB, ECS, AS and Critical Review: SG, MT, MGÇ, SS, HHT, YB, ECS, AS.

### Conflict of interest

The authors disclosed no conflict of interest during the preparation or publication of this manuscript.

### Financing

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