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Original Article

The effects of chard extract against streptozotocin-induced erectile dysfunction in rats

Mustafa Aydın[1], Özlem Saçan[2], Levent Kabasakal[3], Şule Çetinel[4], Mustafa Kadihasanoğlu[5], Muammer Kendirci[6], Yeşim İpçi[3], Refiye Yanardağ[2], Göksel Şener[7]

[1]University of Health Sciences, Department of Urology, Samsun Training and Research Hospital, Samsun, Türkiye
[2]İstanbul University-Cerrahpaşa, Faculty of Engineering, Department of Chemistry, İstanbul, Türkiye
[3]Marmara University, Faculty of Pharmacy, Department of Pharmacology, İstanbul, Türkiye
[4]Marmara University, School of Medicine, Department of Histology and Embriology, İstanbul, Türkiye
[5]University of Health Sciences, Department of Urology, Istanbul Training and Research Hospital, İstanbul, Türkiye
[6]İstinye University Faculty of Medicine, Department of Urology, Liv Hospital Ulus, İstanbul, Türkiye
[7]Fenerbahçe University, Faculty of Pharmacy, Department of Pharmacology, İstanbul, Türkiye

ABSTRACT

Background and Aims: To analyze the potential therapeutic effects of chard against streptozotocin (STZ) -induced erectile dysfunction (ED) and oxidative damage in the corpus cavernousum in rats.

Materials and Methods: In this study, Sprague-Dawley rats (250-300g) were allocated into groups as follows: control, diabetic, diabetic + chard, and diabetic + insulin. In order to induce diabetes, rats were given 65 mg/kg intraperitoneal streptozotocin. Chard extract was given orally at a dose 2 g/kg for 45 days beginning on 15th days. Sixty days after STZ injection, intracavernosal pressure (ICP) was measured and rats were decapitated. Blood samples were obtained for glucose, asymmetric dimethylarginine (ADMA)levels, and lactate dehydrogenase (LDH) activity while cavernous tissues were taken to analyze luminol and lucigenin chemiluminescence (CL), malondialdehyde and glutathione and along with histological analysis.

Results: The results revealed that diabetes caused significant decreases in cavernosal tissue glutathione levels, while luminol and lucigenin CL, and malondialdehyde levels were significantly elevated. Plasma glucose, ADMA levels, and LDH activity were also found to be increased in diabetic group. On the other hand, both chard extract and insulin treatment reversed these biochemical parameters significantly. Furthermore, it was found that the ICP value examined for evaluating erectile functions were lower in the diabetic group, but increased in both treatment groups which were similar to the control values.

Conclusion: According to our results, chard extract, similar to insulin, reduced diabetes-induced oxidative damage in cavernosal tissue and protected erectile functions. This effects may be attributed its hypoglycemic and antioxidant properties.

Keywords: Chard extract, diabetes mellitus, erectile dysfunction, oxidative damage

INTRODUCTION

Increased reactive oxygen species (ROS) increase the risk of atherosclerosis and cardiovascular disease in diabetic patients (Kesavulu et al., 2001).Erectile dysfunction is one of the main complications developing in male diabetes patients and has a negative impact on quality of life. *In vivo* experimental studies report that in diabetic ED, endothelial dysfunction and reduction in nitric acid synthase (NOS) activity are mediated by ROS (Saenz de Tejada, Goldstein, Azadzoi, Krane, & Cohen, 1989; Way & Reid, 1999; Taylor, 2001). A hyperglycemic environment has been linked to increased formation of free oxygen radicals due to glucose auto-oxidation and also protein glycation. When the antioxidant capacity of the body is exceeded, the increased free radicals cannot be scavenge, and in this case, the tissue damage begins due to radicals harmful effects (Hunt, Smith, &Wolff, 1999). The main aim of diabetes treatment is to control hyperglycemia, as well as to prevent complications caused by high blood sugar.

Research on new agents with antidiabetic effects, especially those of natural origin, is still ongoing, and it is also being investigated whether these agents are protective against diabetic complications (Neef, Declercq, & Laekeman, 1995; Grover, Yadav, & Vats, 2002; Gezginci-Oktayoglu, et al., 2014).Chard (*Beta vulgaris* L. var. *cicla*) [Chenopodiaceae] is one of phy-

Corresponding Author: Göksel Şener **E-mail:** goksel.sener@fbu.edu.tr
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totheraupetic and hypoglycemic agents used by diabetic patients in Turkey (Yanardağ & Çolak, 1998). Chard contains phospholipids, ascorbic acid, glycolipids, polysaccharides, folic acid, fatty acids, vitamin E, carotenoids and minerals (Ertik, Sacan, Kabasakal, Sener &Yanardag, 2021). Various Beta vulgaris species contain saponins and flavonoids, which were reported to have hypoglycemic effects (Bozkalfa, Eşiyok, &Kaygisiz Aşçioğul, 2016). Moreover flavonoids, natural polyphenolic substances, can act as antioxidants in biological systems.

In previous studies, hypoglycemic and antioxidant properties of chard extract were studied in various tissues of diabetic rats (Bolkent, Yanardag, Tabakoglu-Oguz, & Ozsoy-Sacan, 2000; Ozsoy-Sacan, Karabulut-Bulan, Bolkent, Yanardag, & Ozgey, 2004; Sacan & Yanardag, 2010; Oztay et al., 2015; Ustundag et al., 2016; Sacan et al., 2018; Tunali, Cimen, & Yanardag, 2020; Ozel et al., 2021). However, the effects of this extract on the cavernous tissue are unknown. In this study, the effect of chard extract on the cavernous tissues of rats with diabetes mellitus with STZ was investigated functionally and biochemically.

MATERIAL AND METHODS

Biological plant material

Chard (*Beta vulgaris* L. var. *cicla*) leaves were obtained from traditional markets in Istanbul and identified by Prof.Dr. Neriman Ozhatay. Chard leaves were rinsed with cold tap water and then washed with cold distilled water in order to remove salts and other contaminants. Leaves were sliced, defoliated at 20 centigrade and then stored in well-sealed plastic bags.

Preparation of aqueous plant extract

Five hundred mL of distilled water was added to the shade-dried and powdered chard leaves (40g) and boiled for 30 minutes. The resulting extract was filtered and the water was removed under pressure. Before administration to rats, extract was dissolved in distilled water.

Animals

Male Sprague-Dawley rats (250-300g) were obtained from Marmara University Experimental Animals Research Center. The ethical committee approval was taken from the Marmara University Animal Care and Use Committee (68.2008.mar). Animals were divided into 4 groups containing 8 rats. Groups were as follows; control, diabetic, diabetic + chard treated, and diabetic + insulin treated.

Diabetes model

Diabetes was established in rats with a single dose and intraperitoneal (ip) administration of 65 mg/kg STZ (Sigma Chemical Co., Louis, MO). STZ was dissolved in citrate buffer, pH 4.5. Rats with plasma glucose levels of 200 to 250 mg/dL after 24 hours were careful diabetic. Chard extract was dissolved in 1 mL of distilled water. Beginning on the 15th day of the experiment, the diabetic + chard group was administered chard extract at a dose of 2 g/kg daily via gavage through an intragastric tube for 45 days, while insulin was given subcutaneously at a rate of 6U/kg/day(Junod, Lambert, Stauffacher, & Renold, 1969).At the end of the experimental period, they were subjected to intracavernosal pressure (ICP) measurement and thereafter decapitated to obtain blood and cavernosal tissue samples.

Plasma assays

An automated analyzer (Bayer Opera Biochemical Analyzer, Germany) was used to detect plasma LDH activities, while ADMA determination was performed with an ELISA kit (Immunodiagnostic AG). Blood glucose levels were measured using glucometer (OneTouch Ultra, LifeScan).

Measurement of erectile responses

On the 60th days of the study, erectile function was evaluated through both ICP and mean arterial pressure (MAP) measurements. Under anaesthesia with pentobarbital (30 mg/kg) trachea was cannulated with PE-240 polyethylene tubing to provide the patency of the airway. MAP in the cannulated left internal carotid artery was measured with an amplifier unit, data acquisition system and Biopac software system. ICP was measured via a 24-gauge needle transducer inserted the left crus of the penis. Isolated cavernosal nerve (CN) stimulation was achieved with a stainless steel bipolar electrode with parallel hooks placed around the nerve. A four-sided rhythm stimulation enthralled CN. Each rat received CNS at a frequency of 15 Hz with a pulse width of 30 seconds. To achieve significant erectile responses, CNS was administered at 2.5, 5, and 7.5 V. The inspiration time was one minute, with a three-to-five-minute rest interval between CNSs. The maximum ICP/MAP ratio is calculated by dividing the recorded highest ICP by the corresponding MAP and expressed as a percentage (Bivalacqua et al., 2003).

Cavernosal tissue malondialdehyde (MDA) and glutathione (GSH) measurement

1 g of tissue after adding 10 mL of 10% TCA was cooled with ice and homogenized in Ultra Turrax tissue homogenizer. Malondialdehyde (MDA) levels in tissue were determined by thiobarbituric acid method (Beuge & Aust, 1978). Cavernosal tissues were homogenized in cold 0.9% NaCl with glass equipment to obtain 10% (w/v) homogenate. Glutathione levels in tissue homogenate were analyzed according to the modified Ellman procedure (Beutler & Gelbart, 1986).

Luminol and lucigenin chemiluminescence (CL) measurements

Luminol and lucigenin chemiluminescence measurements are the best indicators of the amount of ROS formed in the tissues. Junior LB 9509 luminometer (EG&G Berthold, Germany)was used and measurements were done at room temperature (Haklar et al., 2002).

Histological assay

Tissues were washed in water after 10% formalin was applied and water was removed with increasing alcohol concentrations. It was then incubated in paraffin at 60°C overnight and the next day the tissue was embedded in paraffin blocks. The 6 mm thickness tissue sections embedded in paraffin were subjected to a series of treatments, colored with Hematoxylin-Eosin dyes and examined under the light microscope.

Statistical analysis

Data was analyzed using GraphPad Prism 5.0 statistical program (GraphPad Software, San Diego, CA, USA). Groups were compared with analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. $p<0.05$ was considered statistically significant.

RESULTS

Blood glucose, body weight and ADMA values, and LDH activities of the groups were summarized in Table 1. Blood glucose values were found to be significantly higher in the diabetic rats while chard extract treatment decreased the glucose levels. Although insulin treatment also decreased the glucose levels; it was still higher than the control. ADMA level and LDH activity were found to be increased due to diabetes induction but both treatments decreased ADMA level and LDH activity significantly. Diabetes caused significant increase in both luminol and lucigenin CL and MDA levels in corpus cavernosum. On the other hand, GSH levels were decreased in the diabetic group ($p<0.05$-0.001). Chard extract and insulin treatment significantly reversed the elevations in the luminol, lucigenin CL and MDA levels, while reduced GSH levels were returned to the control levels ($p<0.05$-0.001) (Fig.1).

Erectile responses were assessed in control, diabetic, diabetic + chard and diabetic + insulin groups in a voltage dependent manner by ICP measurements. The ICP trace obtained with stimulation of the CNS at a setting of 7.5 V for 1 minute is shown in Figure 2. The mean ICP and ICP/MAP values recorded after CNS stimulation for 1 minute in all groups were given in Figure 3. In diabetic rats, these values were found to be significantly lower than in control, diabetic + chard and diabetic + insulin groups. On the other hand, chard treatment restored the erectile responses significantly ($p<0.05$-0.001).

In histological examination, regular cavernous structures were observed in the control group. Moderate irregularities and fibrotic structures were observed in the cavernous structures in the diabetic group and slight damage was observed in the endothelial structures. The damage in corpus cavernosum regressed indiabetic + chard and diabetic + insulin groups (Fig.4).

DISCUSSION

The results of the present study demonstrate that diabetes causes oxidative tissue damage in the corpus cavernosum of the rats, as evaluated by augmented luminol and lucigenin CL and lipid peroxidation, and reduced GSH levels. Treatment with chard extract reduces oxidant production thereby lipid peroxidation, and refills GSH content in the tissues, confirming the possible defensive effect of chard in contradiction of oxidative injury. Furthermore, chard extract protecting cavernosal tissue, erectile dysfunction seen in diabetic group was also improved. Since ED that is caused by diabetes significantly affects quality of life in male patients, chard extract may be an alternative option to prevent diabetic complications.

In the past, the vast majority of ED was thought to be due to nonspecific physiologic causes, but nowadays 50-80% of the affected males have detected organic etiology (Bivalacqua, Champion, Hellstrom, & Kadowitz, 2000). Organic ED is most often linked to vascular risk factors such as arteriosclerosis, hyperlipidemia, hypertension, and diabetes mellitus. ED is more common in diabetic men than in the normal population and treatment is often more difficult this subgroup (Rendell, Rajfer, Wicker, & Smith, 1999). Currently, there are various medications and medical applications in ED treatment. However, the treatment of ED in diabetic patients should be related to diabetes induced ED pathophysiology, which may increase the treatment success. Furthermore, when side effects of drugs in ED treatment are taken into account, plant-based treatment alternatives are even more remarkable.

Chard (*Beta vulgaris* var. *cicla*) is one of the phytotherapeutic used as another hypoglycemic medicine by diabetic patients in Turkey. The hypoglycemic effect of chard was initially described by Yanardağ & Çolak (1998). Experimental studies have shown that the chard regenerates pancreatic beta cells, protects kidney functions in diabetic animals, and prevents oxidative stress caused by hyperglycemia in skin, heart, and aorta (Tunali et al., 1998; Sener, Saçan, Yanardağ, & Ayanoğlu-Dülger, 2002; Yanardağ, Bolkent, Özsoy-Saçan, & Karabulut-Bulan, 2002). Therefore, chard can be used as an alternative treatment for type 1 and type 2 diabetes due to its regarenative effect in pancreatic beta cells and its hypoglycemic effect with saponins and flavonoids (Bozkalfa et al., 2016).

ADMA, is an endogenous inhibitor of nitric oxide synthase

Table 1. Blood glucose, body weight and ADMA levels and LDH activities of all groups.

	Control	Diabetic	Diabetic+chard	Diabetic+insulin
Glucose (mg/dL)				
t0	91 ± 8.1	90 ± 5.2	94 ± 6.3	95 ± 7.1
24th hours	95 ± 5.1	310 ± 30.6 ***	331 ± 21.7 ***	333 ± 24.1 ***
15th days	96 ± 5.3	371 ± 31.3 ***	351 ± 26.3 ***	383 ± 23.4 ***
60th days	97 ± 5.4	362 ± 25.5 ***	143 ± 9.8 $^{+++}$	164 ± 14.2 *, $^{+++}$
Body weight				
t0	311 ± 5.3	310 ± 9.4	322 ± 8.6	309 ± 8.2
60th days	405 ± 6.3	289 ± 6.9	404 ± 7.1	419 ± 10.5
ADMA (µmol/L)	1.03 ± 0.1	3.42 ± 0.3 ***	1.65 ± 0.2 $^{+++}$	2.08 ± 0.2 *, $^{++}$
LDH (U/L)	76 ± 4.2	133 ± 10.5 ***	95.8± 5.1 $^{++}$	103± 7.2 $^{+}$

Each group consists of 8 rats. Values are represented as mean ± SEM.
* p<0.05, *** p<0.001; vs control group. $^{+}$p<0.05, $^{++}$p<0.01^{+++}p<0.001; vs vehicle-treated diabetic group.

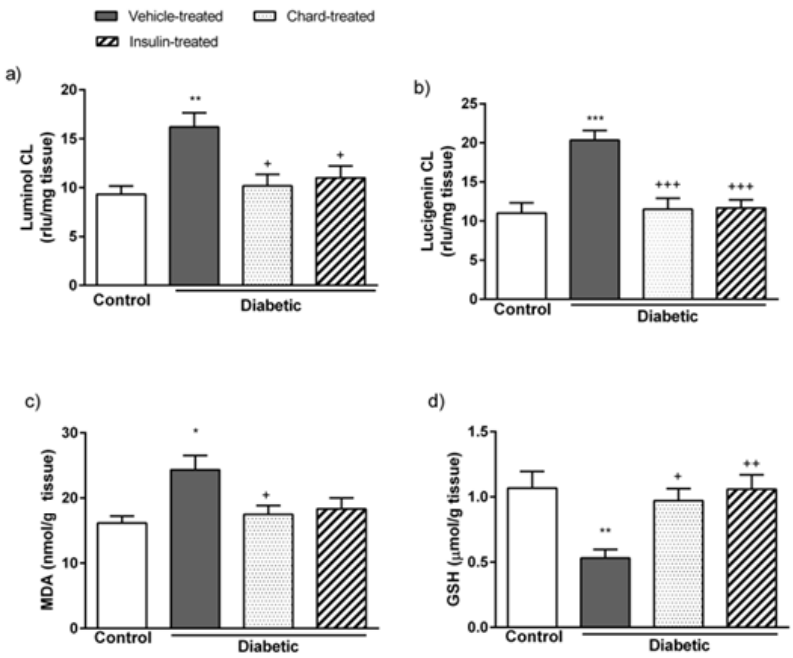

Figure 1. a) Luminol CL, b) Lucigenin CL, c) Malondialdehyde and d) Glutathione (GSH) values of control, diabetic, diabetic + chard and diabetic + insulin groups.Standards are signified as mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001; vs control group. $^{+}$p<0.05, $^{++}$p<0.01^{+++}p<0.001; vs vehicle-treated diabetic group.

(NOS) and augmented plasma levels of ADMA causes endothelial and thereby cardiovascular dysfunction. However, the relation of ADMA to diabetes, glycemic control, and renal function, specially early diabetic hyperfiltration, remains unknown (Kielsteinet et al., 2003). Asymmetric dimethylarginine activating renin-angiotensin system and NAD(P)H oxidase leads to increase in production of $O2^{-\bullet}$, which then delays with the bioavailability of NO, subsequent in reduced dilation and augmented arteriolar tone (Veresh, Racz, Lotz, & Koller, 2008). Therefore, increased ADMA levels in our study may have led to reduce NO bioavailability and may have led to erectile dys-

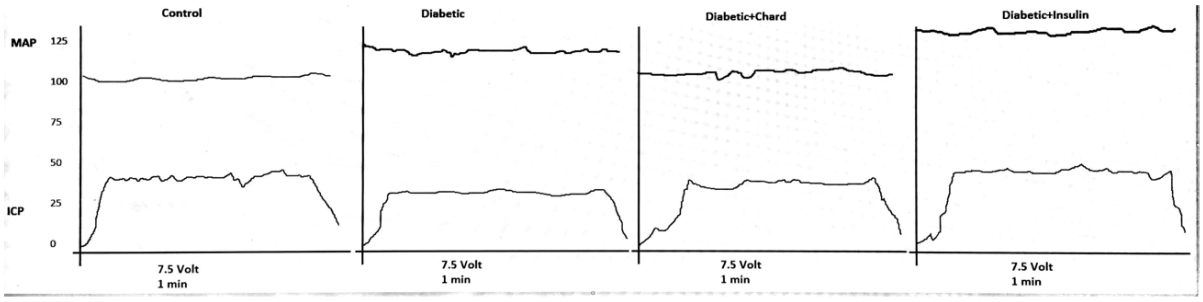

Figure 2. Representative ICP and MAP tracing after CNS at 7.5 V setting for 1 minute in control, , diabetic, diabetic + chard and diabetic + insulin groups.

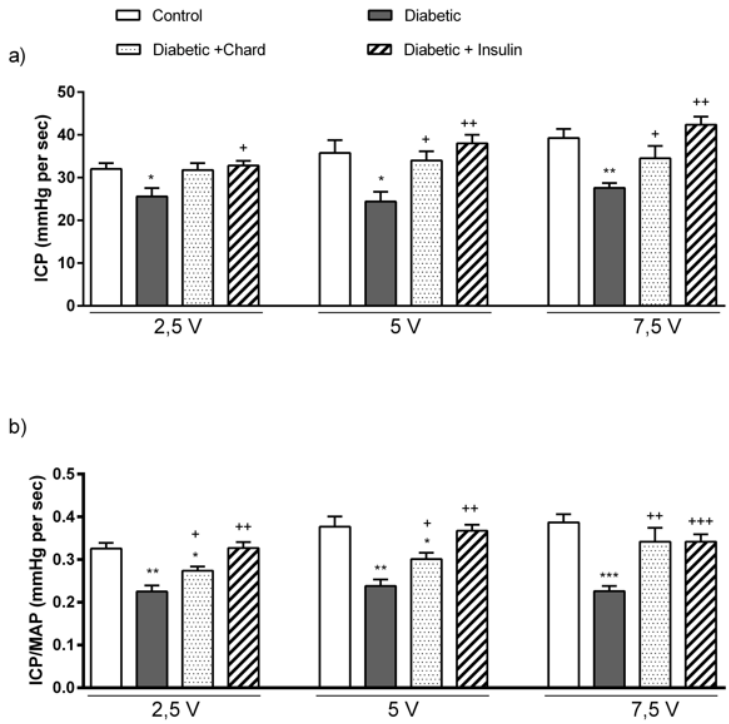

Figure 3. a) ICP and b) ICP/MAP values of control, diabetic, diabetic + chard and diabetic + insulingroups. Values are represented as mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001; vs control group. $^{+}$p<0.05, $^{++}$p<0.01, $^{+++}$p<0.001; vs vehicle-treated diabetic group. (ICP: Intracavernosal pressure, MAP: Mean arterial pressure).

function. In contrast chard extract, suppressed ADMA levels and thus protected erectile function.

Increased ROS levels in diabetic human corpus cavernosum tissue compared to controls may designate that ROS formation theatres a role in the pathogenesis of diabetic endothelium and ED (Seftel et al.,1997). In the present study oxidant making was evaluated with luminol and lucigenin CL measurements. Luminol notices a group of reactive species, i.e. hypochlorous acid (HOCL), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-),while lucigenin is discerning for superoxide anion($O2^-$) (Haklar et al., 2002). Our results demonstrated that diabetes increased these oxidant species in the cavernosal tissue since both CL values were significantly increased. On the other hand, chard extract effectively reduced the production of oxidants and there by lipid peroxidation. Free radicals, which are promoted by increased protein glycosylation and glucose auto-oxidation, may increase lipid peroxidation in some organs. A significant increase in the level of MDA from lipid peroxidation products resulting from oxidative stress in the erectile tissues of diabetic rats and a decrease in the level of GSH responsible for antioxidation of free radicals has been reported (Ryu et al.,2003). Similar to Ryu et al. study, in our study, lipid peroxidation product MDA increased in diabetic animals and GSH levels responsible

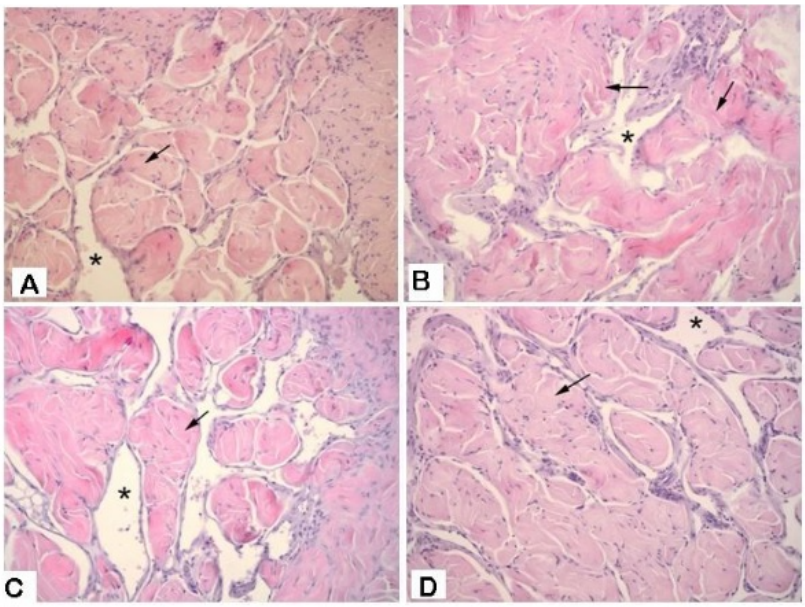

Figure 4. Regular cavernous structures were observed in the control group (A). Regular cavernous structure (arrow) and endothelium (*). In diabetic group (B) moderate irregularities and fibrotic structures (arrows) were observed in cavernous structures and slight damage (*) was observed in endothelial structures. The damage in corpus cavernosum regressed in diabetic + chard (C) and diabetic + insulin (D) groups. Cavernous structure (arrow), regular endothelium (magnification, X200).

for antioxidation decreased significantly. This result confirms the characterization of diabetic corpus cavernosum with overexpression of free radicals and inadequate elimination. In this study, chard treatment increased GSH levels significantly in diabetic rats. In parallel with this finding, the lipid peroxidation in diabetic + chard group was significantly lower than the diabetic group ($p<0.05$). Also, this value was similar to the control group. This finding showed the strong antioxidative activity of chard in diabetes mellitus. Chard extract treatment reduced oxidative stress by showing antioxidant activity in the penis as in other tissues, but also significantly improved erectile answer to cavernous nerve stimulation in vivo.

The overproduction of free radicals and the attenuation of antioxidant systems competing with them may be responsible for the structural and functional damage of the corporal endothelium and smooth muscle responsible for ED (Ryu et al., 2003). Ahn et al. (2005) showed that Transforming Growth Factor-β1 (TGF-β1) expression and corporal fibrosis increased in diabetic rats, and fibrosis regressed with phosphodiesterase type 5 (PDE5) inhibitor treatment. In present study, moderate irregularity and fibrotic structures were observed in the cavernous structures in the diabetes group and moderate damage to the endothelial structures was observed. It was observed that the damage was regressed with both chard and insulin treatment (Ahn et al., 2005). Moreover, this information supports the therapeutic use of antioxidants or free-radical protectants in diabetic ED.

Men with diabetic ED are a difficult group to treat and are often resistant to treatment with standard oral PDE5 inhibitors. In the general population, the effect of PDE5 inhibitors is shown between 70-89%; however, the effect is just over 50% in the diabetic population (Rendell et al.,1999). One of the possible causes of this relative resistance has been reported to be oxidative stress (De Young, Yu, Freeman, & Brock, 2003). It has been reported that a significant increase in sildenafil response was observed in diabetic rats given vitamin E (De Young, Yu, Freeman, & Brock, 2004). In our study, chard showed an antioxidant effect, in addition to the normoglycemic effect. Based on these data, antioxidant treatments such as chard may be useful as an alternative treatment in patients with diabetic ED. In addition, this study is the first in the literature to evaluate the effects of chard on the penile tissue. Further studies are needed to confirm these findings.

CONCLUSIONS

The results of this study indicated that chard extract exhibited protective activity against diabetes-induced erectile dysfunction. This effect may be related with augmented activity of anti-oxidant protection system and decrease in oxidative stress in the penile corporal cavernousa of rats. Chard leaf extract with having strong antioxidant properties, can protect the cavernosal tissue against diabetes-induced oxidative damage and thus regulate erectile dysfunction.

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ORCID IDs of the authors

Mustafa Aydın	0000-0002-4183-6045
Özlem Saçan	0000-0001-6503-4613
Levent Kabasakal	0000-0002-5474-6436
Şule Çetinel	0000-0003-1912-3679
Mustafa Kadihasanoğlu	0000-0001-5109-5319
Muammer Kendirci	0000-0002-1854-1871
Yeşim İpçi	0009-0003-6476-8799
Refiye Yanardağ	0000-0003-4185-4363
Göksel Şener	0000-0001-7444-6193

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