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EXPERIMENTAL STUDY

Protective effect of distillated Nerium oleander on heart of type 2 diabetic rats

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ABSTRACT
OBJECTIVES: The current study aims to investigate the possible role of NO distillate either for therapeutic or for protective potential in diabetic cardiomyopathy.
BACKGROUND: Protective and restorative effects of distillated *Nerium oleander* (NO) on the diabetes-induced electrophysiological and structural alterations were investigated.
METHODS: Type 2 diabetes was induced by combination of single dose streptozotocin injection and high fat diet for four weeks. Experimental groups were designed as follows: control, diabetic, restorative-NO treated diabetic and protective-NO treated diabetic. Intracellular action potentials (AP) and contractile activities were measured form left ventricular papillary muscle strips as well as histopathological examination of heart tissue and biochemical examinations of serum were performed.
RESULTS: Type 2 diabetes induced AP prolongation was prevented with both ways of NO treatments. Moreover, treatments produced nearly complete restorations of diabetes-induced depressed amplitude and altered kinetics of contractile activities. In parallel to electrophysiological parameters, both histopathological and biochemical results indicates the NO induced beneficial effects on the diabetes related alterations.
CONCLUSION: Distillated Nerium oleander (NO) can be a highly potential therapeutic or preventive agent on the diabetes induced excitation-contraction coupling alterations *(Tab. 3, Fig. 3, Ref. 23)*. Text in PDF *www.elis.sk.*
KEY WORDS: type II diabetes, cardiomyopathy, Nerium oleander, heart, electrophysiology.

Introduction

Non-insulin-dependent diabetes mellitus (NIDDM) (diabetes mellitus type 2) is a metabolic disorder that is characterized by high blood glucose in the context of insulin resistance and/or relative insulin deficiency. Diabetes must take its place alongside the other major risk factors (stroke, peripheral arterial disease, nephropathy, retinopathy, and possibly neuropathy) as important causes of cardiovascular disease (CVD), more specifically *diabetic cardiomyopathy*. It is a unique entity unassociated with coronary artery disease and it was concluded that it was also a distinct entity characterized by diastolic dysfunction, which was commonly associated with hypertension and/or with myocardial ischemia. Several factors probably underlie diabetic cardiomyopathy: severe coronary atherosclerosis, prolonged hypertension, chronic hyperglycemia, micro vascular disease, glycosylation of myocardial proteins, and autonomic neuropathy. Improved glycemic control, better control of hypertension, and prevention of atherosclerosis with cholesterol-lowering therapy may prevent or mitigate diabetic cardiomyopathy.

Nerium oleander (NO) is an evergreen flowering shrub that belongs to the dogbane family: Apocynaceae. The parts of NO that contain cardiac glycosides are collectively called as nerine and oleandrin (1). Cardiac glycosides, naturally occurring in plant or animals, have beneficial and toxic effects on the routine work of heart (2). Besides their toxicity NO have been used in the past as folklore medicine for the congestive heart failure disease (3, 4). It has been reported that the leaves of NO possess cardiotonic (5, 6), hypolipidemic (7), hypoglycemic (8) and cardio-protective agent for oxidative stress induced changes (9). Although the hypolipidemic and hypoglycemic potential of NO have been screened, there have been no reports on the possible therapeutic and/or protective effect of NO on the diabetic cardiomyopathic changes. Therefore current study aims to investigate the possible role of NO distillate (375 μg/0.5 ml dH_2O/day) on the diabetic cardiomyopathy (both for therapeutic and for protective potential).

Methods and materials

Obtaining a Lyophilized NO

NO plant was collected among new shoots in March-September period from Mediterranean region of Turkey, identified and

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authenticated at the department of biology. After washing the collected plant, fresh shoots were chopped, and distilled water (100 g NO plant/1000 ml water) added. NO distillate was obtained by the heat of the mixture in heat resistant container. Then, NO distillate was lyophilized in small glass bottle (20 ml) by using lyophilizator (Patent No: WO/2010/082906 Use of Nerium oleander for diseases manifested with type ii diabetes, obesity, high cholesterol and triglyceride). Lyophilized NO distillate was dissolved at the concentration of 750 µg/ml in distilled water.

Induction of diabetes and experimental groups

Experiments were carried out after having Ethical Committee approval (Selcuk University Ethics Committee of the Faculty of Veterinary Medicine, Konya, Turkey, report no: 2010/058). In this study, 40 Sprague Dawley male rats (12 weeks of age) were used for the experiments. The experimental animals were housed in polysulfone cages, and heat (22 ± 2 °C), light (12/12 hour light/dark) and humidity (55 ± 5 % relative humidity) were controlled.

Animals were randomly divided into four groups each of which included 10 rats. For the induction of type 2 diabetes (for three groups), streptozotocin (single STZ 35 mg/kg, which was dissolved in citrate buffer (pH 4.5), intraperitoneally) was administered together with the high fat diet for two weeks (3). One week after STZ injection, rats with ≥ 300 mg/dl non-fasting blood glucose level were considered to be type 2 diabetic. The control group animals received only citrate buffer. The treatment and the diet content of experimental animal groups were as follows; Control group animals (Con): Healthy control rats fed with normal pellet diet and did not have the NO distillate; Diabetic group animals (DM): Type 2 diabetic rats fed with high fat diet and did not have NO distillate; NO treated diabetic animals (OZ): Type 2 diabetic rats fed with high fat diet. They received the NO distillate at a dose of 375 µg/0.5 ml of distilled water by gavages once in a day from thirtieth day of diabetes induction to the end of the experiment; Prophylactic NO treatment to diabetes (POZ) Type 2 diabetic rats fed with high fat diet. They received the NO distillate at a dose of 375 µg/0.5 ml of distilled water by gavage once in a day to the end of the experiment starting from the induction of diabetes.

Biochemical evaluations of the experimental animal groups

Body weight changes of each animal were noted weekly. Fasting blood samples were taken from tail vein of all rats at 30 day intervals through the experiment. Blood samples were centrifuged (3000 g for 10 min) to separate serum. Serum samples were analyzed immediately for fasting blood glucose (FBG), Aminotransferase (AST); Cholestrol (CHOL) and Triglyceride (TG) by using commercially available colorimetric diagnostic kits (IL TestTM, Instrumentation Laboratory, Milano, Italy).

Histopathological examinations of the experimental animal groups

The excised hearts were rinsed with saline solution to remove excess blood and clots and placed into 10 % neutral buffered formalin solution. Whole mount sagittal sections including all four cardiac chambers were performed. The specimens were processed in an automatic tissue processor over night and embedded in paraffin blocks. 4 micron thickness sections were obtained and stained with Hematoxylin Eosin stain for routine light microscopic morphologic evaluation. According to the evaluated morphologic criteria, extra histochemical and immunohistochemical stains were performed. Toluidine Blue, Masson Trichrome, and Connexin stains were performed according to manufacturer's data sheet respectively.

Action potential and contraction recordings

Under the intramuscular ketamine (35 mg/kg) and xylazine (5.0 mg/kg) anesthesia, hearts were excised rapidly. Papillary muscles of the left ventricle were excised under binocular microscope, placed in a recording chamber, and pinned down at one end with a stimulating electrode while the second end was connected to a force-displacement transducer (MAY FDT 05). The dimensions of the papillary muscle strips were similar between the groups. The recording chamber was superfused with Krebs solution (gassed with 95% O_2–5% CO_2 and maintained at 37 °C) mM: 119 NaCl, 4.8 KCl, 1.8 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 18 $NaHCO_3$, and 10 glucose (pH 7.4). Intracellular action potential recordings were performed with glass microelectrodes, which were manufactured from glass capillaries (Clark Electromedical Instruments) with a puller (PT 30, Sutter Instrument Co). The microelectrodes were filled with 3 M KCl had 15–18 MΩ resistance and were connected to a microelectrode amplifier (IE-251A, Warner Instrument Co.). The muscle strips were stimulated with rectangular electrical pulses 3 ms in duration at a frequency of 0.2 Hz (MP150, Biopac Systems Instruments). Simultaneous recordings of the action potential and contractions were performed 20 min after the equilibration period. The action potential and contraction data were transferred to a PC through an MP150 data acquisition system for further analysis. Unless otherwise specified, measured parameters compared with Data are presented as the mean ± SEM throughout the text. Differences between the experimental groups were assigned by one way analysis of variance. Values in which $p < 0.05$ were accepted as significant.

Results

General and biochemical characteristics of the experimental animals.

Mean body weights, the blood glucose levels and the measured biochemical parameters of the experimental groups are summa-

Tab. 1. General characteristic of the experimental animals.

	BW (g)		BG (mg/dL)	
	Start	End	Start	End
CON (n=10)	231.17±5.85	353.83±7.98*	99.33±3.63	100.17±4.39
DM (n=10)	229.31±5.56	331.00±6.72*	443.00±35.33α	435.00±23.87α
OZ (n=10)	232.86±7.24	346.86±9.55*	497.50±32.48α	393.67±26.56α
POZ (n=10)	240.85±9.39	340.83±12.56*	466.45±25.60α	385.33±24.23α

In the table: Control (Con), Type 2 diabetic (DM), Oral Nerium Oleander supplemented type 2 diabetic (OZ) and prophylactic Nerium oleander supplemented type 2 diabetic (POZ) group of animals. Body weights and blood glucose are represented as BW and BG respectively. * represents the degree of significance ($p < 0.05$) compared to mean starting values. α represents the degree of significance ($p < 0.05$) compared to control group of animals. Values are presented as the mean ± SEM.

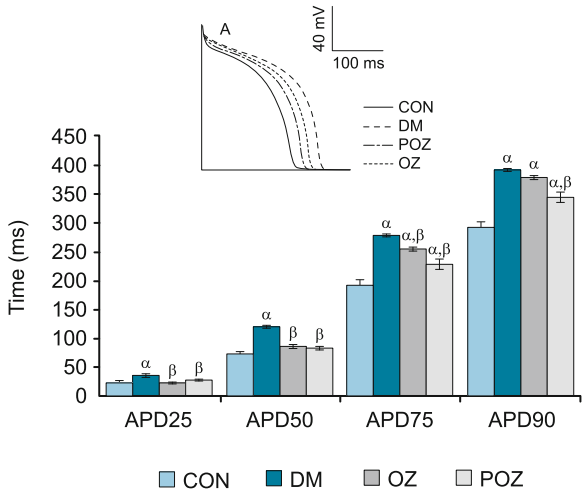

Fig. 1. Action potential parameters of the experimental group of animals. Control (Con), Type 2 diabetic (DM), Oral NO supplemented type 2 diabetic (OZ) and prophylactic NO supplemented type 2 diabetic (POZ) group of animals. A shows original action potential traces. In part B the APD 25; 50; 75; 90 represents the time required to reach the 25, 50, 75, and 90 % of the repolarization values, respectively. Values are presented as the mean ± SEM. $^{\alpha}$ represents the degree of significance ($p < 0.05$) compared to control group animals while $^{\beta}$ represents the degree of significance ($p < 0.05$) compared to diabetic group of animals.

rized in Table 1. All experimental groups animals gained weight during the experimental period. Blood glucose levels of both non-treated (DM) and NO treated diabetic groups (OZ and POZ) were found to be significantly higher than the control group animals. Although the treatment did not produce statistically significant results, the measured BG values resembled a decrement in the treated diabetic groups. At the end of the 4-weeks of diabetic period mean plasma aminotransferase, cholesterol and triglyceride levels were found to be increased (Tab. 1). Both of the treatment types of NO in diabetes were found to restore these measured values to control group animal values.

Histopathology

Histopathological results of the heart tissue obtained from the experimental groups are summarized in Table 2. All of the pathologic and morphologic criteria described above were observed in the diabetic group in all 7 subjects except interstitial fibrosis which was observed in only 3 subjects. When compared with the control group, the most widespread finding in this group was alteration and disorganization of Z lines. It was observed in all subjects and graded as 2+. Loss of myocyte contact, myocyte necrosis, replacement fibrosis, cytoplasmic vacuolization were also observed in all subjects but the surface extent percentage was less. When compared with the diabetic group, OZ group also showed Z line alterations in all 7 subjects but this time the surface extent percentage was less and the alterations were not widespread but only focal. Cytoplasmic vacuolization was only observed in 2 subjects as least surface extend. Likewise, loss of myocyte contact was observed only in one subject; myocyte

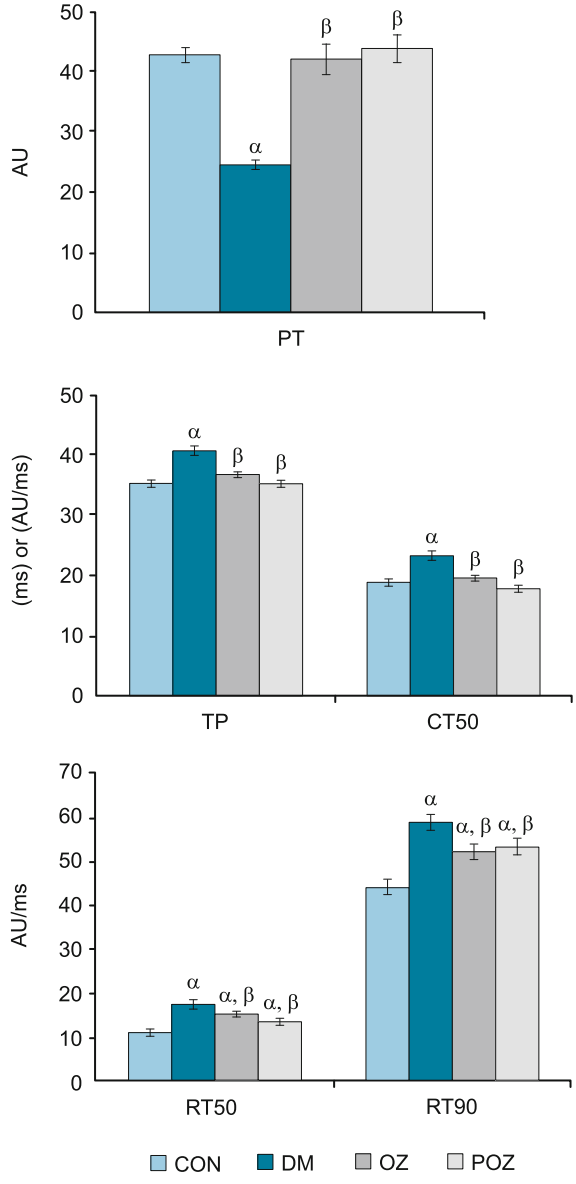

Fig. 2. Contraction parameters of the experimental group of animals. Control (Con), Type 2 diabetic (DM), Oral NO supplemented type 2 diabetic (OZ) and prophylactic NO supplemented type 2 diabetic (POZ) group of animals. A shows the peak tensions (PT) of the experimental group of animals in arbitrary units. B shows the average values of the time to peak and the average contraction kinetics of the experimental groups. C shows two points mean relaxation kinetic values (50 and 90 %). Values are presented as the mean ± SEM. $^{\alpha}$ represents the degree of significance ($p < 0.05$) compared to control group of animals while $^{\beta}$ represents the degree of significance ($p < 0.05$) compared to diabetic group of animals.

necrosis in 4; replacement fibrosis in only 1 subject. Interstitial fibrosis was not observed in this group. In POZ group, only the cytoplasmic vacuolization was improved and observed in one subject when compared with OZ and DM groups. Results of alteration of Z lines were the same with OZ group but definitely improved when compared with DM group. Although focal myo-

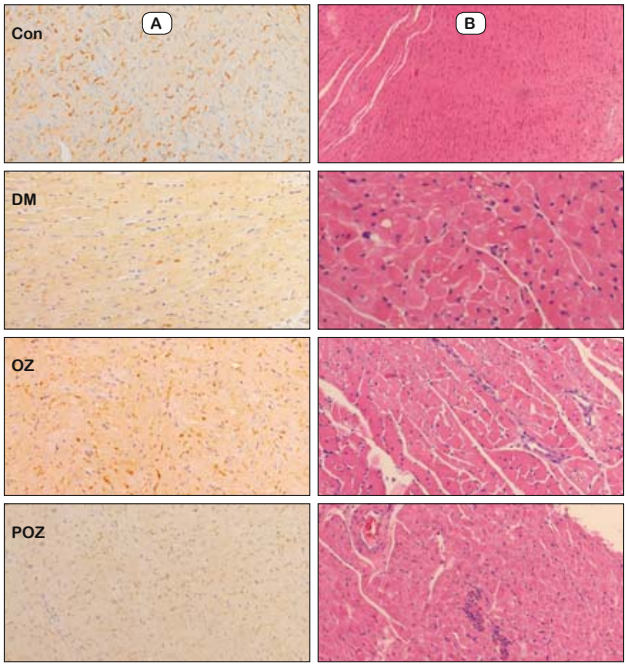

Fig. 3. Samples from immunohistochemistry results of the experimental group of animals. Control (Con), Type 2 diabetic (DM), Oral NO supplemented type 2 diabetic (OZ) and prophylactic NO supplemented type 2 diabetic (POZ) group of animals. In column A connexin staining samples from the groups. In column B Hematoxylin Eosin staining from the experimental groups.

cyte necrosis was present, replacement fibrosis was not observed in this group.

Connexin staining was graded as mild, moderate and intense. The control group showed diffuse and intense connexin expression at the intercalated discs as vertical lines and horizontal linear staining indicating the positivity between the adjacent myofibrils (Fig. 3). Only faint and sparse expression of connexin at intercalated discs was observed at DM group, and horizontal staining in between the adjacent cardiac myofibrils was absent. OZ and POZ groups showed significant moderate expression of connexin at intercalated discs and mild horizontal expression in between the adjacent myofibrils showing the improvement caused by NO. However, staining intensities did not differ significantly between OZ and POZ groups.

Tab. 2. Biochemical parameters of the experimental group of animals.

	AST	CHOL	TG
Con (n=10)	117.67±2.92	102.67±3.30	43.17±2.45
DM (n=10)	153.60±6.26$^{\alpha}$	129.29±8.96$^{\alpha}$	246.14±97.10$^{\alpha}$
OZ (n=10)	90.29±4.53$^{\beta}$	99.71±7.41$^{\beta}$	124.43±19.25$^{\alpha,\beta}$
POZ (n=10)	88.67±5.67$^{\beta}$	99.83±2.88$^{\beta}$	92.17±15.18$^{\alpha,\beta}$

In the table: Control (Con), Type 2 diabetic (DM), Oral Nerium Oleander supplemented type 2 diabetic (OZ) and prophylactic Nerium oleander supplemented type 2 diabetic (POZ) group of animals. Open forms of the representations are: Aminotransferase (ALT); Cholestrol (CHOL); Triglyceride (TG). $^{\alpha}$ represents the degree of significance ($p < 0.05$) compared to control group of animals while $^{\beta}$ represents the degree of significance ($p < 0.05$) compared to diabetic group of animals. Values are presented as mean ± SEM.

Effect of NO treatment on action potential parameters

At the end of the experimental period, no change was found between the rapidly excised papillary muscle's excitation related values (Time to peak (TP) and Resting membrane potential (RMP)). Mean measured TP values (ms): $TP_{Con} = 4.90 \pm 0.07$; $TP_{DM} = 4.89 \pm 0.16$; $TP_{OZ} = 4.90 \pm 0.07$; $TP_{POZ} = 4.90 \pm 0.09$ and RMP values (mV): $RMP_{Con} = -84.39 \pm 2.24$; $RMP_{DM} = -88.58 \pm 1.51$; $RMP_{OZ} = -85.35 \pm 1.60$; $RMP_{POZ} = -82.68 \pm 2.18$.

The effect of both the diabetes and the NO applications on the time courses of the action potential repolarization phases are shown in Figure 2. APD25;50;75;90 represents the time required to reach the 25, 50, 75, and 90 % of the repolarization values, respectively. In parallel to current literature diabetes it ended up with an increase in all of the measured repolarization values. Both the protective (POZ) and treatment application (OZ) to diabetes subjects resulted in a complete restoration of APD25 and 50 values. Except for the OZ group's APD90 values, mean APD75 and 90 values have shown a significant shortening with the NO application (Fig. 1B).

Effect of NO treatment on contraction

Figure 2 shows the effect of NO treatment on the mechanical activities of the papillary muscle. Figure 2A shows the amplitudes of the peak tension (PT) measured in arbitrary units. The maximum contractile performances of the papillary muscle strips were significantly lower in the diabetes group. The mean values of NO treated diabetic groups (OZ and POZ) were found to be similar to control group values (Fig. 3A). Together with the decreased PT, the time required to reach the PT was greater in the diabetic group (Fig. 3B). Here, again the NO treated diabetic group values were found to be normalized to control group values. Diabetic group showed significant increase in the contraction kinetics (CT50) and both applications of NO reversed this diabetes induced alterations to control group animal values. Parameters related to relaxation period of contractions are summarized in Figure 3C. Like the contraction phase, relaxation periods were found to be increased with diabetes and NO application reversed this adverse effect. But the effect seen on mean RT50 values was more pronounced than RT90 values (Fig. 3C).

Discussions

Diabetic cardiomyopathy, independently of the type, is one of the leading secondary complication seen in diabetes. Indeed, it increases the incidence of patient's mortality and morbidity seen in diabetes in longer periods of exposure to diabetes. Experimental models of diabetes are the most preferred method for studying diabetes induced secondary complications since they produce similar complications within shorter periods of times.

Current study showed that NO supplementations (either for treatment or for protective purposes) prevented type II diabetes induced altered lipid profile and cardiomyocytes dysfunction (Tab. 2 and Figs 1 and 2). Several mutually reinforcing factors such as increased triglycerides and cholesterol levels account for the increased CVD risk among patients with diabetes. For the majority of cases, treatment strategies include medication to control the

Fig. 3. Samples from immunohistochemistry results of the experimental group of animals. Control (Con), Type 2 diabetic (DM), Oral NO supplemented type 2 diabetic (OZ) and prophylactic NO supplemented type 2 diabetic (POZ) group of animals. In column A connexin staining samples from the groups. In column B Hematoxylin Eosin staining from the experimental groups.

	Con (n=6)		DM (n=7)		OZ (n=7)		POZ (n=7)	
Parameters	1+	2+	1+	2+	1+	2+	1+	2+
Loss of myocyte contact	–	–	7	–	1	–	2 (f)	–
Myocyte necrosis	2 (f)	–	7 (mf)	–	3 (f)	1 (mf)	2 (f, mf)	–
Replacement fibrosis	–	–	7 (+)	–	(1)	–	–	–
İnterstitial fibrosis	–	–	3	–	–	–	2 (f)	–
Vascular sclerosis	–	–	7 (+)	–	–	–	–	–
Perivascular fibrosis	–	–	7	–	–	–	–	–
Alteration of Z lines	–	–	–	7	7 (f)	–	7 (f)	–
Cytoplasmic vacuolisation	3 (f)	–	3(mf) 2(f)	2	2 (f,mf)		1 (mf)	–

In the table Control (Con), Type 2 diabetic (DM), Oral Nerium Oleander supplemented type 2 diabetic (OZ) and prophylactic Nerium oleander supplemented type 2 diabetic (POZ) group of animals are represented by abbreviations. Quantization of the parameters: + and – sings represents the presence and absence respectively. Numbers correspond to the presences of variables among the total experimental animal numbers. During the examination of the myocardial tissue if the parameter was seen in one focal area, then 'f' is used but if the parameter was seen more than one focal area (Multifocal) then mf is used as abbreviation.

altered lipid profile of the patients. Although the mechanism of action underlying beneficial effect of controlled lipid profile is poorly understood, it has been evident that the lowered plasma triglyceride levels improve the cardiac pump function in diabetic hearts. Previously we have shown that NO extracts can be used to regulate the lipid metabolism in diseases manifested with type 2 diabetes and obesity (Patent No: WO/2010/082906 Use of Nerium Oleander for Diseases Manifested with Type 2 Diabetes, Obesity, High Cholesterol and Triglyceride).

NO extract as a potential therapeutic agent (OZ group animals) results in shortening of the diabetes induced longer repolarization phases (RP) of action potentials (APD25;50;75 and 90). This effect is more pronounced in early phases of the RPs (APD25 and 50) indicating a potential modulation on the transient outward current (I_{to}) and L type calcium channel currents (I_{CaL}). Prolongation of these phases can be due to suppression of the I_{to} and/or increased I_{CaL}. There are many studies suggesting that these ionic currents have been affected in the animal models of diabetes (10, 11, 12, 13). Applications of cardiac glycosides to healthy normal cardiomyocytes on the other hand results not only in shortening of the measured APD values but also resulted in a decrement in the measured I_{CaL} in a time dependent manner (14, 15). Among the many other explanations (changes in the steady state inactivation or decrease in maximum conductance of the channel), reduction in the calcium equilibrium potential can be the best answer for why the L type calcium currents were reduced with the cardiac glycosides application.

The results obtained from human and the animal diabetes model studies resemble a malfunction in the free cellular calcium handling which leads to reduced contractility (16, 17, 18). Our contraction recordings showed a decreased contractile force, increased time required to reach peak contraction as well as increased both contraction and relaxation kinetics (Figs 2 A, B and C). Here for the first time we showed that NO applications both for the therapeutic (OZ) and for the protective (POZ) purposes result in a complete restoration on the contractile force and contractile kinetics. In fact, this application also results in promising results in the relaxation periods of the contractions. Positive effects were seen in the kinetic data indicating that NO reversed the calcium induced calcium release mechanism which was altered by diabetes. Meanwhile, reuptake mechanisms, which were altered by diabetes, were found to be recovered by NO application. The increased fraction of exchangeable calcium during treatment with cardiac glycosides is brought about by a redistribution of intracellular calcium, possibly by mobilization of otherwise tightly bound calcium (19). Another possible explanation for the NO induced normalization can be due to reduction in the myocardial calcium content which was shown by other topic oriented researches (20, 21).

Under physiological conditions, transaminases are the cellular enzymes increased plasma levels of which are the main indicators for cellular damage. Increased plasma AST levels may be attributed to the heart tissue damage as well as to predict incidents of type 2 diabetes, insulin resistance, metabolic syndrome, and CVD (22, 23). In a supportive manner to our histopathological results increased AST levels results have been found to be significantly reduced with the application of NO either for treatment or for protective purposes.

The current study has for the first time shown that NO applications for both treatment and protective purposes have promising positive effects on diabetes induced alterations. The detailed molecular mechanisms of these positive effects, either on the basis of ionic currents or on the structural ones, need further investigations.

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