Antioxidant Treatments Improve Diabetes Induced Endothelium-Dependent Vascular Dysfunction

Antioksidan Tedavi Diyabetle Indüklenen Endotel Bağımlı Vasküler Disfonksiyonu Düzeltir

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Abstract
Purpose: Diabetes is an important cardiovascular risk factor and impaired endothelium-dependent relaxation has been demonstrated in vascular beds of different models of diabetes. Hyperglycemia causes important changes in cells due to increased production of reactive oxygen species (ROS) and different types of receptor-mediated vasorelaxation can change during diabetes.
Material and Methods: In our previous studies we have shown that antioxidant treatment improved diabetes-induced alterations in heart function. Therefore, in this study we aimed to evaluate the effect of treatment with either sodium selenate (0.3 mg/kg/day, intragastrically) or a long chain (n-3) polyunsaturated fatty acid enriched with vitamin E (omega-3E; 50 mg/kg/day, intragastrically) on mechanical function of streptozotocin (STZ)-induced diabetic rat aortas for 4-week.
Results: Both types of treatments improved the impaired relaxation responses to acetylcholine (ACh; 10-9–10-4 M) of endothelium-intact (pre-contracted with phenylephrine; 10-6 M) aortas. Furthermore, these treatments prevented diabetes-induced decreased level of cAMP production with ACh stimulation.
Conclusion: Taken together, our data demonstrate that these beneficial effects of both sodium selenate and omega-3E on the mechanical properties of the diabetic aortas appear, in part, to be related to inhibition of ROS production and regulation of cell antioxidant state arised in diabetic vascular dysfunction.

Key Words: **Selenium; Long chain (n-3) fatty acid; Aorta; Relaxation; Muscarinic Receptors; cyclic AMP.**

Özet
Amaç: Kardiyovasküler hastalıklar için diyabet hastalığı önemli bir risk faktör olup, diyabetin endotel-bağımlı çeşitli damar örneklerinin gevşeme yanıtlarında bozulmalara neden olduğu farklı diyabet modellerinde gösterilmiştir. Kan şekeri yüksekliği (hyperglycemia) hücrelerde reaktif oksijen türlerinin (ROS) artmasına neden olabilmekte ve ayrıca farklı tiplerde reseptör-aracılı damar gevşeme yanıtlarının değişmesine neden olabilmektedir.
Gereç ve Yöntemler: Daha önceki çalışmalarımızda antioksidan tedavisinin, diyabetin neden olduğu kalp fonksiyon bozukluklarında düzelmeye neden olduğunu gösterdiğimiz için bu çalışmamızda da sodyum selenatın (0.3 mg/kg/gün, intragastrik yolla) ve karşılaştırmak amacıyla vitamin E ile zenginleştirilmiş uzun-zincir doymamış-yağ asiti bileşiğinin (omega-3E; 50 mg/kg/gün, intragastrik yolla), streptozotosin (STZ) ile diyabet yapılmış sıçanların aortalarının mekanik fonksiyonları üzerine etkisini araştırmayı amaçladık.
Bulgular: Her iki tip uygulama sonucunda da, diyabetik sıçanlarda asetilkolin(ACh; 10^{-9}–10^{-4} M) uyarımıyla gerçekleşen endotel-bağımlı gevşeme bozukluklarının düzelme gösterdiği tespit edilmiştir (10^{-6} M fenilefrinle ön kasılma sağlanmıştır). Ayrıca bu tedavilerin diyabetik aortalarda ACh uyarımıyla ölçülen düşük cAMP üretim değerlerini düzelttiği gözlenmiştir.
Sonuç: Bu veriler bir bütün olarak değerlendirildiğinde, hem sodyum selenatın hem de omega-3E'nin ROS üretiminin engellenmesi ve hücresel antioksidan durumunun düzenlenmesi yoluyla diyabetik aortaların mekanik fonksiyonlarının düzelmesinde etkili oldukları anlaşılmaktadır.

Anahtar Kelimeler: **Aorta; Muskarinik Reseptörler; Uzun Zincirli Yağ asidi; Gevşeme; Selenium; Siklik AMP.**

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Introduction

Diabetes Mellitus (DM) is associated with vascular complications in many organs and increased mortatility and morbidity due to its impact on the cardiovascular system. Diabetes Mellitus causes a number of well-characterized morphological changes of the cardiovascular system (1). In an early study by Sullivan and Sparks (2), diminished contractile responses to norepinephrine of aortas from diabetic rats were demonstrated. In diabetic animals, trace elements were found to have positive effects on the parameters of glucose metabolism (3, 4). In our previous studies, we have demonstrated that both sodium selenite treatment and omega-3E (a long chain (n-3) polyunsaturated fatty acid enriched with natural vitamin E) prevented the diabetes-induced ultrastuructural and functional changes of the heart isolated from diabetic rats, and reversed the increased platelet aggregation and thromboxane B_2 level to the control levels (5-7).

In many types of smooth muscles, including vascular smooth muscle, β-adrenoceptor (β-AR) agonists cause relaxation responses and are known to exert their relaxing effects through the production of cAMP (8). The relaxation of vascular smooth muscle induced by β-AR stimulation also has been demonstrated to be decreased in a variety of hypertensive animals as well as diabetics (9,10). It has been proposed that vascular endothelial cells, which utilize non-insulin-mediated mechanisms for glucose transport, are the primary targets of glucosed-induced damaged (see review by 11). Hemodynamic studies have indicated that endothelium-dependent vasodilatory response in diabetics is impaired (12,13). The exact mechanism of these diabetes-induced vasculature changes has not been elucidated, oxidative stress has been postulated to be a major contributor to the pathogenesis of these events (see reviews by 11, 14, 15).

Experimental evidence demonstrated that one of the prominent cellular changes occurred in the cardiovascular system of a diabetic animal is an imbalance between prooxidants and antioxidants (16, 17). Besides different mechanisms involved in the genesis of the oxidative stress in diabetic status, there is also evidence that Diabetes Mellitus can depress natural antioxidant defense agents such as glutathione (16) and lower antioxidant defense system (17). It is known that several types of antioxidants, including selenium and vitamin E, inhibit atherosclerosis and improve endothelial function, mostly affecting oxidative stress in animal models (18-23). Due to the potent antioxidant properties of tocopherols, the impact of α-tocopherol in the prevention of chronic diseases, especially those believed to have an oxidative stress component, has often been studied, and beneficial effects have been demonstrated (21-24). Furthermore, the effects of a long chain (n-3) polyunsaturated fatty acid from fish in heart diseases obtained epidemiological studies, animal and in vitro experiments were discussed widely in recent two review articles (25, 26). Increasing evidence related with increased consumption of long chain (n-3) polyunsaturated fatty acids benefits subjects with Diabetes Mellitus without adversely affecting glucose control has prompted the American Diabetes Association (2002) to recommend the consumption of fish. In this regard, we demonstrated that administration of a long chain (n-3) fatty acid, similar to an antioxidant, selenium had beneficial effect as a therapeutic adjuvant in the treatment of diabetic cardiomyopathy (6, 27).

Therefore, it is important to study the effect of antioxidants such as sodium selenate and a long chain (n-3) polyunsaturated fatty acid enriched with vitamin E (omega-3E) therapy on signalling pathways in the endothelium-dependent relaxation responses of diabetic rat aortas. We examined the changes in the basal vascular function and the responses to muscarinic receptor stimulation of diabetic rat thoracic aorta.

Materials and Methods

Animals and experimental design. Male rats (200–250Êg) were divided into two groups. Diabetes Mellitus was induced by a single intraperitoneal injection of streptozotocin (STZ, 50 mg/kg body weight and dissolved in 0.1 M citrate buffer, pH 4.5) into a tail vein (diabetic group; DM group) while the control group (CON group) received comparable volumes of vehicle (citrate buffered saline), daily. One week after injection of STZ, blood glucose levels were measured using a glucose analyser (Glucotrend, Roche) and rats with blood glucose at least 4-fold higher than pre-injection levels were used as the diabetic (DM) group. Diabetic rats were divided into three groups and received the following treatments: selenium group (DMSE group)- daily sodium selenate (0.3 mg/kg, intragastrically), omega-3E group (DMOE group)- daily omega-3E (50 mg/kg/day, intragastrically), and DM group- daily saline (intragastrically) for 4-week. All rats had free access to water and standard rat chow during the experimental protocol. Since we did not observe any effect on any function of the rats from CON group which were fed with either sodium selenate or omega-3E, we did not include any data related with these groups (5-7). This

investigation conforms to the guide of animal care and experimental procedure in accordance with Ankara University ethics guidelines (No: 2007-11-39).

Vasoactive responses in isolated thoracic aorta preparations. Diabetic and control (nondiabetic) rats were anesthetized with pentobarbital sodium (30 mg/kg body weight, intraperitoneally) and thorax was opened. The thoracic aortas were removed and placed immediately in ice-cold Krebs solution as described previously (28). The aortas were cleaned of the adhering perivascular tissue, cut into 3-mm width rings. The rings were opened by a single cut and then fixed with stainless steel clips at both ends in organ baths of 10 mL volume containing warmed (37C) and aerated (95% O_2 and 5% CO_2) Krebs solution (in mmol/L: 110 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, and 11 dextrose in double-distilled water at pH 7.4, bubbled with 95% O_2 and 5% CO_2). Isometric contractions were measured using force-displacement transducer (Grass FT 03) and a preamplifier (May-Com, Ankara) connected to a personal computer. Before starting the experiments, the resting tensions of the aortic rings were adjusted to 1-1.5 g and allowed to equilibrate for 1 hour.

Following equilibration for 1 hour, relaxant responses of aortas to muscarinic receptor agonist (acetylcholine; ACh) were determined in phenylephrine (Phe; 10^{-6} M)-precontracted endothelium-intact tissues. The concentration-response curves of contractions were obtained by using cumulatively increasing concentration of ACh (10^{-9}-10^{-4} M).

The maximum responses to ACh and the negative logarithm (log EC_{50}; a measure of tissue sensitivity) were calculated from the concentration-response curves in which these two parameters are the most important two parameters used for the characterization of the differences between the two curves.

cAMP assay. Cycylic AMP (cAMP) levels of the samples were measured as decribed previoulsy (29). Isolated aortic strips were placed in microcentrifuge tubes containing 250 µl of Dulbecco's modified Eagle's medium with 250 U/mL penicilin/streptomycin and isobutylmethylxanthine (IBMX, 1 mmol/L). The reaction was started by adding agonist (ACh; 10^{-6} M) for 10 minutes at 37°C. After the incubation HCl (0.2 N final concentration) was added to sample mixtures to stop the assay. The tubes were then left on ice for 1 hour. Then 10 µL of the samples were used to measure cAMP levels. Accumulated cAMP was measured by radioimmunoassay using acetylation protocol. Accumulation of cAMP per mg tissue per minute was considered as adenylate cyclase activity.

Pharmacological agents. Sodium selenate (Sigma), omega-3E (kindly gift from FreeFlow, Vesteralens Naturprodukter AS, Sortland-Norway), phenylephrine (phenylephrine hydrochloride, Sigma) and acetylcholine (acetylcholine chloride, Sigma) were used in the assessment of vascular function. All other chemicals used were also purchased from Sigma (Sigma-Aldrich Chemie, Steinheim, Germany).

Statistical analysis. Groups were tested and compared using ANOVA and Tukey post-hoc test. P values <0.05 were taken as significant, and significance levels were given in the text. Data are presented as mean ±SEM, throughout the text.

Results

General effects of selenium or omega-3E treatment on the diabetic rats. The general features of the rats from the four groups are summarized in Figure 1. All rats injected with STZ developed severe diabetes at the end of one week as indicated by increased blood glucose levels (1. week). Blood glucose level was at least 4-fold higher in DM group compared to those of the CON group (Table I). Both sodium selenate and omega-3E treatment of the diabetic rats caused small but significant decrease in the high blood glucose level ($p<0.0001$). Rats from the DM group did lost weight, while control rats as well as treated rats (DMSE and DMOE groups) continued to gain weight over the 5-week experimental period (Table I).

Table I. Changes in body weight and blood glucose levels.

Groups/Parameters	Weight (g)		Blood Glucose (mg/dL)	
	1.week	5. week	1.week	5. week
CON (n=12)	262±7	287±8	112±7	113±12
DM (n=12)	259±6	189±13*	487±15	481±18*
DMSE (n=13)	256±6	274±10	484±8	424±10*†
DMOE (n=11)	258 ±8	297 ±9	490 ±6	430 ±8*†
F:		210.6		327.3
p:		0.0001		0.0001

"1. week" and "5. week" represent the observation times at the first and fifth weeks following streptozotocin injection during the experimental period, respectively. CON; control group, DM; diabetic group, DMSE; sodium selenate-treated diabetic group, and DMOE; omega-3E-treated diabetic group.Values are expressed as mean SEM. Tukey post-hoc test: *p<0.0001 *vs* CON group; †p<0.0001 *vs* DM group; n number of rats.

Since both sodium selenate and omega-3E treatments did not affect any parameters of the rats from control group (5-7), we, in here, did not measure any parameters related with thoracic aorta.

Protective effects with either sodium selenate or omega-3E treatment against diabetes-induced vascular contractile dysfunction. Basal tensions (as mg) of the aortic preparations were similar in the four group (Fig. 1 A). The maximum responses of the DM group to both KCl (80 mM) and Phe (10^{-6} M) stimulations were significantly ($p<0.05$) less than that of the CON group while both treatments prevented these declines, significantly ($p<0.05$). Since we obtained less response to the Phe stimulation in the DM group compared to the CON group although their basal tensions were similar, the relaxation responses of each group were calculated as the % relaxation of its maximum response of the individual group.

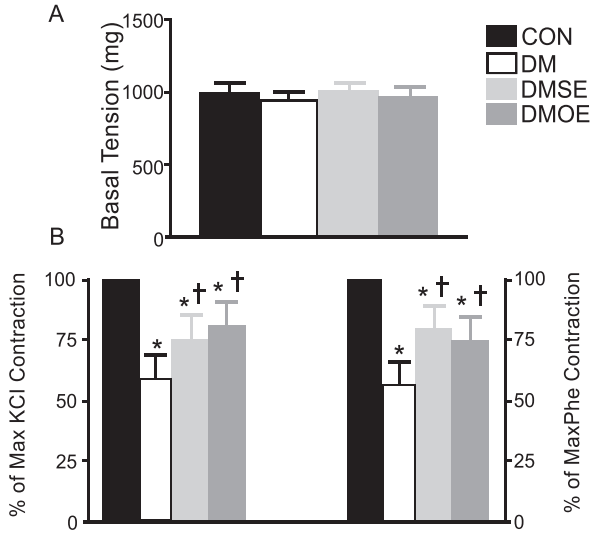

Figure 1. Preservation of diabetes-induced depression in the contractile responses of thoracic aortas with either sodium selenate or omega-3E treatment. A: Basal activities of the endothelium-intact thoracic aortic rings. B: Percentage maximal contractile responses to KCl (80 mM) or Phe (10^{-6} M) stimulations. Bars represent mean SEM. *P<0.05 *vs* CON group, †P<0.05 *vs* DM group.

Protective effects with either sodium selenate or omega-3E treatment against diabetes-induced endothelial-dependent vascular dysfunction. The effects of two different antioxidants, sodium selenate and omega-3E treatments of diabetic rats on endothelium-mediated responses of aortas were evaluated by measuring the relaxation of endothelium-intact aortic rings in response to a muscarinic-receptor, ACh. As shown in Fig. 2, ACh evoked concentration-dependent relaxation of the Phe-precontracted preparations. The maximum relaxation of the preparatinos to ACh was significantly ($p<0.05$) reduced in the DM group relative to CON group (Fig. 3 A) with a significant change in the log EC_{50} values to ACH (-6.4 ± 0.1 *vs* -6.8 ± 0.1). Both Sodium selenate and omega-3E treatments showed significant ($p<0.05$) protection against diabetes-induced reduced response to ACh of endothelium-intact aortic rings as weel as on the log EC_{50} (Fig. 3 B).

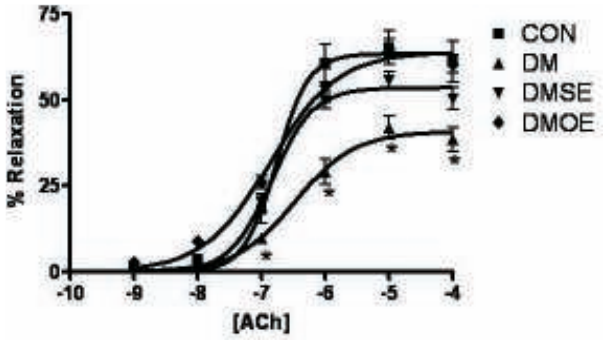

Figure 2. Effects of sodium selenate and omega-3E treatments on diabetes-induced impairment of relaxation functions of endothelium-intact aortic rings. Concentration-response curves obtained as percentage relaxation responses of each group with respect to its Phe (10^{-6} M) precontraction to ACh in endothelium-intact thoracic aortas. Bars represent mean SEM. *P<0.05 *vs* CON group.

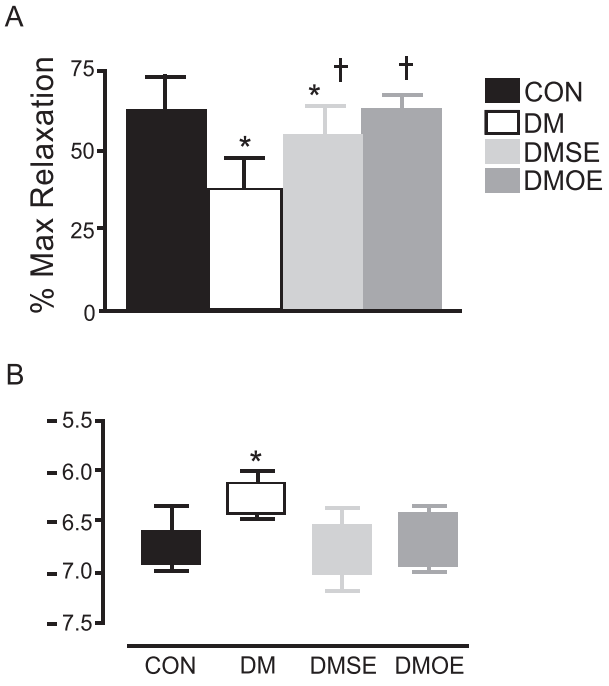

Figure 3. Maximum relaxation responses and their $logE_{50}$ values. A: Maximum relaxation responses of each group (as percentage) with respect to its Phe (10^{-6} M) precontraction to ACh stimulation in endothelium-intact thoracic aortas. B: Their $logE_{50}$ values calculated from the concentration-response curves of each experimental group. Bars represent mean SEM. *P<0.05 *vs* CON group, †P<0.05 *vs* DM group.

Effect of sodium selenate or omega-3E treatment on cAMP accumulation. Basal cAMP levels of the aortic preparations from the four groups were found similar (Table II). Acetylcholine (ACh; 10^{-6} M)-stimulated cAMP accumulation was 4.3-fold less in the endothelium-intact aortic rings of diabetic rats compared to those of the controls. Sodium selenate or mega-3E treatment of the diabetic rats caused significant (p<0.0001) protection in that of less response to ACh stimulation in the diabetics. The effects of these two different antioxidant treatments on ACh-stimulated cAMP accumulation were in the same order in those aortic preparations.

TableII. Effects of antioxidant treatments on basal and ACh-stimulated cAMP levels.

Groups	Basal cAMP (pmol/g tissue/min)	ACh-stimulated cAMP (pmol/g tissue/min)
CON (n=12)	3.68 ± 0.89	26.70 ± 3.83
DM (n=12)	3.15 ± 0.76	6.18 ± 0.41*
DMSE (n=13)	3.95 ± 0.36	11.92 ± 0.65*†
DMOE (n=11)	4.2 ± 0.2	12.7± 0.92*†
F	2.745	121.5
p:	0.07	0.0001

Values are expressed as mean SEM. CON; control group, DM; diabetic group, DMSE; sodium selenate-treated diabetic group, and DMOE; omega-3E-treated diabetic group. Tukey post-hoc test: *p<0.0001 *vs* CON group; †p<0.001 *vs* DM group; n number of rats.

Discussion

This study demonstrated that treatment with either sodium selenate or omega-3E significantly reduceed diabetes-induced vascular dysfunction. Moreover, these beneficial effect was observed not only in the contractile responses but also in the relaxation responses of the endothelium-intact aortic samples. Functionally this reveals that the endothelium-dependent component of diabetes-induced vascular dysfunction can be mediated by increased ROS and oxidant stress. Clinical studies have consistently found that endothelium-dependent vasodilation in patients with Diabetes Mellitus (30, 17) which is fully coincided with the knowing physiological facts on the role of nitric oxide (NO) and hyperglycemia in endothelial cell functions as well as in vascular smooth muscle cells. Hyperglycemia causes PKC activation and subsequent ROS production via NAD(P)H oxidase in both endothelial and smooth muscle cells, which in turn to vascular dysfunction in terms of contraction and relaxation of the vessels (31, 32). All these hypothesis are fully supported with our data presented in here. We demonstrated that the vascular dysfunction of endothelium-intact thoracic aortas is induced by increased levels of oxidative stress due to high levels of hyperglycemia, which also oxidized the protein free and total protein sulfhydryl (SH)'s and caused alteration in NO levels. Therefore, treatment of diabetic rats for 4-week with either sodium selenate or omega-3E preserved these alterations significantly. Consequently, we obtained a normal mechanical activity of aortic rings after these antioxidant treatments compared to the untreated diabetics.

These present results support the hypothesis that treatment with selenium compounds is beneficial for diabetic samples and arised reduction in cardiovascular mortality (23). This is the first data that sodium selenate treatment improved diabetes-induced depressed endothelial-dependent relaxation function of aortic rings mainly due to the increased oxidation levels of protein thiols and decreased endothelial nitrate level. This treatment also caused significant improvement of the responses of aortic rings to the -AR agonist stimulations. Complications associated with type 1 Diabetes Mellitus primarily represent vascular dysfunction that has its origin in the endothelium. As it has been already realized in all aspects of cardiovascular physiology during the past 20 years, normal endothelial cells maintain a delicate balance in the vascular between vasoconstriction and vasodilatation (33). While many of the vascular changes are more accountable in the latest stages, changes that occur in the early or initial functional stages of this disease might be associated with the observed decreases in vascular NO availability with the development of type 1 Diabetes Mellitus. At the onset of Diabetes Mellitus, there is selective dysfunction of receptor- and endothelium-dependent agents that lead to a reduced release or response to NO (34). Evidence associating ROS with the etiology of diabetic complications in the vasculature is founded to be increased generation of ROS and/or decreased endogenous antioxidant defense mechanisms in diabetic endothelial dysfunction. ROS may impair endothelial function through inactivation of NO or by serving as an endothelium-derived contracting factor (34).

Vascular tone is driven by a dynamic process in which several signals that are either cell-mediated or receptor-mediated are involved. A mojor role is played by vascular adrenoreceptors, and this effect is coupled to the regulatory role played by the endothelium through mediator release. This complex and finely tunes interplay is distrupted by diabetes resutlting in endothelium dysfunction. It has been shown that blood vessels isolated from diabetic animals exhibited an attenuated endothelium-dependent relaxation (35). Lam et al. (36) have demonstrated that impaired endothelim-dependent relaxation of diabetic mice aorta was not due to changes in the protein levels of eNOS and M3 receptors. Our results showed also that muscarinic-receptor agonist ACh-mediated vasorelaxation responses were depressed significantly in the diabetic rats. These results, in part, are in line already published data in which it was shown that decreases in ACh-mediated vasorelaxation responses were associated with the fact that muscarinic receptors are affected by diabetes. Furthermore, our cAMP data are also demonstrating a diabetes-induced marked depression in ACh-stimulated cAMP production of the arotic preparations. Moreover, these functional findings and biochemical data yet need further studies to understand the underlying mechanisms of these beneficial effects with these two different types of antioxidants. Therefore, our present data are supported with already known early hypothesis related with vascular disorders associated with both metabolic defects and early changes in vascular reactivity to vasoactive neurotransmitters in experimental diabetic animals (11).

Several antioxidants have been studied in diabetic animals with respect to their ability to correct vascular dysfunction (37). Indeed, it has been demonstrated that new superoxide dismutase or catalase mimetic compounds normalized endothelial dysfunction in STZ-induced diabetic rats (38) which is providing in vivo data to support an important functional role of ROS in diabetic vascular disease. In summary, these data suggest that drug therapies aimed at inhibiting either oxidative stress and/or ROS production may be a useful strategy to protect vasculature undergoing oxidative stress during chronic diseases such as Diabetes Mellitus.

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