ELSEVIER

Archives of Medical Research 38 (2007) 503–511

Archives of Medical Research

ORIGINAL ARTICLE

Effects of Stobadine and Vitamin E in Diabetes-Induced Retinal Abnormalities: Involvement of Oxidative Stress

Fatma Yülek,[a] Meral Or,[a] Candan Özoğul,[b] Asli Ceylan Isik,[c] Nuray Ari,[c] Milan Stefek,[d] Victor Bauer,[d] and Cimen Karasu[e]

[a]*Department of Ophthalmalogy,* [b]*Department of Histology*
[e]*Medical Pharmacology, Faculty of Medicine, Gazi University, Besevler, Ankara, Turkey*
[c]*Department of Pharmacology, Faculty of Pharmacy, Ankara University, Tandogan, Ankara, Turkey*
[d]*Institute of Experimental Pharmacology, Slovak Academy of Sciences, Bratislava, Slovak Republic*[1]

Received for publication November 23, 2006; accepted February 20, 2007 (ARCMED-D-06-00499).

Background. Because hyperglycemia-induced oxidative stress may be a cause of retinopathy, this study examined the hypothesis that administration of exogenous antioxidants, stobadine (ST) and vitamin E (vitE), can restore retinal abnormalities in experimental diabetes.

Methods. Normal and streptozotocin (STZ)-induced male Wistar rats received daily intraoral doses of ST (24.7 mg/kg) and vitE (α-dl-tocopherol acetate, 400–500 IU/kg) individually or in combinations for 8 months. The biochemical parameters including aldose reductase enzyme (AR) activity and lipid peroxidation (MDA), and histopathological changes such as retinal capillary basement membrane thickness (RCBMT) and vascular endothelial growth factor (VEGF) expression were evaluated.

Results. A 37.99% increase in RCBMT was observed in rats after 8 months diabetes duration. The increase in RCBMT was 12.34% in diabetic rats treated with ST and 23.07% in diabetic rats treated with vitE. In diabetic rats treated with antioxidant combination, just a 4.38% increase was observed in RCBMT. The excess VEGF immunoreactivity and increased MDA and AR activity determined in diabetic retina were significantly attenuated by individual antioxidant treatments. Although both antioxidants decreased blood glucose, HbA1c, fructosamine and triglyceride levels in diabetic rats, poor glycemic control was maintained in all experimental groups during the treatment period. However, the antioxidant combination led to almost complete amelioration in retinal MDA and RCBMT in diabetic rats.

Conclusions. The ability of antioxidant combination to arrest retinal abnormalities and lipid peroxidation even in the presence of poor glycemic control might advocate the key role of direct oxidative damage and the protective action of antioxidants in retinal alterations associated with diabetic retinopathy.

Key Words: Diabetic rat, Stobadine, Vitamin E, Lipid peroxidation, VEGF, Aldose reductase, Retina.

Introduction

Retinopathy is the most common complication of diabetes mellitus, affecting up to 90% of diabetic patients and progressing to loss of vision in about 5% of cases (1,2). Hyperglycemia, of course, is the initiating event in the development of retinopathy. However, studies have clearly shown that the oxidative and nitrative modifications in retina occur early in

[1]Antioxidants in Diabetes-Induced Complications, The ADIC Study Group.

Address reprint requests to: C. Karasu, Department of Medical Pharmacology, Faculty of Medicine, Gazi University, Besevler, Ankara, Turkey; E-mail: karasu@gazi.edu.tr

0188-4409/07 $–see front matter.
doi: 10.1016/j.arcmed.2007.02.006

the course of development of retinopathy in diabetes, and these abnormalities are not easily reversed by the reinstitution of good glycemic control (3). In addition, the reinstitution of normal glycemic control after a period of poor glycemic control has been shown to be unable to produce immediate benefits on the progression of retinal changes (3). Due to the fact that tight metabolic control is often difficult to achieve and to maintain in diabetic patients, supporting therapies need to be involved in order for diabetic retinal alterations to be prevented and/or delayed (4).

The retina is the neurosensorial tissue of the eye and is extremely rich in polyunsaturated lipid membranes. This feature makes it especially sensitive to oxygen- and/or nitrogen-activated species and lipid peroxidation. According to this, a correlation between increased serum lipid hydroperoxides and the prevalence of diabetic retinopathy has already been demonstrated (5,6). In addition to triggering oxidative/nitrosative stress, hyperglycemia is involved in the pathogenesis of diabetic retinopathy via multiple mechanisms such as increased activation of aldose reductase (AR) (7) and protein kinase C (PKC) (8), as well as elevated nonenzymatic glycation and glycooxidation of proteins (9). Among the proposed pathogenic mechanisms, the polyol pathway model has received the most scrutiny. AR is the first enzyme in the polyol pathway, converting excess glucose to sorbitol, which is then further metabolized to fructose by sorbitol dehydrogenase. According to several studies, AR is responsible for the early events in the pathogenesis of diabetic retinopathy, leading to a cascade of retinal lesions including blood-retinal barrier breakdown, loss of pericytes, neuroretinal apoptosis, glial reactivation, and neovascularization (7). Increased AR activity has been shown to contribute to increased oxidative stress by promoting nonenzymatic glycation and the activation of PKC (10). It has been demonstrated that AR inhibition counteracts diabetes-induced oxidative and nitrosative stress and prevents vascular endothelial growth factor overexpression, basement membrane thickening, pericyte loss, and microaneurysms in retinal capillaries (11–13). In long-term diabetes-induced neuroretinal stress, increased expression of VEGF and apoptosis and proliferation of blood vessels have been shown to be less prominent in AR-deficient animals (14,15). Sustained hyperglycemia-induced hypoxia and retinal ischemia also lead to an increase in vasoactive growth factors and their receptors that initiate retinal angiogenesis. Many angiogenic factors have been incriminated in the development and progression of diabetic retinopathy, and basement membrane thickening is an important property indicating the progression of retinopathy occurring in parallel with other lesions such as acellular nonperfused capillaries and pericyte loss (16).

Due to the fact that retinopathy remains one of the primary causes of vision loss and blindness in diabetic patients, recently more targeted pharmacological prevention and treatment strategies altering several metabolic pathways have been developed. Among these therapeutic interventions, some antioxidant compounds, AR, AGE, PKC and VEGF inhibitors, are largely investigated in diabetic animals and patients (17). In this context, it has been clearly demonstrated that administration of various antioxidants are able to inhibit the development of the early stages of diabetic retinopathy (18,19). A strong association between hyperglycemia-induced retinal oxidative stress and the development of retinal histopathology has been supported by studies in diabetic animals treated with multiple antioxidants (18–23). Dietary supplementation with a comprehensive mixture of antioxidants inhibits apoptosis, activation of NF-κB, nitric oxide and nitrotyrosine formation in retinal endothelial and microvascular cells and pericytes of diabetic animals (21–24). Moreover, both oxidative stress and the accumulation of advanced glycation end products have been discussed together in the promotion of retinal apoptosis. In this context, advanced glycation end-product inhibitors (OPB–9195) (24), a transition metal ion chelator and dicarbonyl scavengers (Tenilsetam) (25), omega-3 long-chain polyunsaturated fatty acids (EPA, DHA) (26), pigment epithelium-derived factor (27), angioprotective agents (calcium dobesilate) (28) have all been shown to be useful for the treatment of early diabetic retinopathy.

ST [(-)-cis 2,8 dimethyl 2,3,4,4a,5,9b hexahydro-1H-pyrido-(4,3b-indole)] is an antioxidant molecule produced by the Slovak Academy of Sciences (29). ST scavenges hydroxyl, peroxyl and alkoxyl radicals, quenches singlet oxygen, repairs oxidized amino acids and preserves oxidation of SH groups by one-electron donation. ST has been reported to be effective in the prevention or treatment of neurodegenerative and cardiovascular diseases (29–31). Although the beneficial effects of ST have also been observed in metabolic, cardiovascular, renal, neural, and hepatic systems in diabetes mellitus (29–32), its effects on diabetic retina have not been studied previously. On the other hand, a lipophilic antioxidant vitE (α-tocopherol) inhibits lipid peroxidation, scavenging lipid peroxyl radical to yield lipid hydroperoxides and the tocopheroxyl radical. VitE supplementation has been shown to ameliorate biochemical, metabolic and histopathological retinal abnormalities caused by diabetes (19,22,24).

In the present study we studied the effect of ST treatment on the thickening of retinal capillary basement membrane (RCBM), lipid peroxidation (MDA), aldose reductase enzyme (AR) activity and vascular endothelial growth factor (VEGF) immunoreactivity in long-term streptozotocin (STZ)-diabetic rats and compared the effects of ST with the effects of vitE in those animals.

Materials and Methods

Animals and Treatments

Male Wistar-Albino rats (Ankara University Research Laboratory, Ankara, Turkey) weighing 250–300 g were

assigned randomly to be made diabetic or to remain as normal controls. Diabetes was induced by an IP injection of STZ (55 mg/kg) dissolved in citrate buffer (pH 4.5). Ten days after STZ administration, all animals with plasma glucose level >250 mg/dL (Glucometer Elite; Bayer Corporation, Tarrytown, NY) were considered diabetic and were included in the study. The rats did not receive insulin during the study. Some of the diabetic and control rats were treated with vitE (α-dl-tocopherol acetate, 400−500 IU/kg/day) and/or ST-dipalmitate (ST) (obtained from the Slovak Academy of Sciences, Department of Experimental Pharmacology) and made into a suspension with 0.2% carboxy methylcellulose, 24.7 mg/kg/day by intraoral gavage, whereas some did not have antioxidant supplement. The treatment dose of ST-dipalmitate was chosen according to previous observations (31,32). Six groups with ten rats in each were evaluated: control group without antioxidant treatment (C), control group with vitE plus ST supplementation (CEST), diabetic group without antioxidant treatment (D), diabetic group treated with vitE (DE), diabetic group treated with ST (DST) and diabetic group with vitE plus ST supplementation (DEST). Animals were treated for a period of 8 months beginning 10 days after either vehicle or STZ injection. Principles of laboratory animal care (NIH publication no. 85−23, revised 1985) were observed.

Blood Measurements

After 8 months, body weights and plasma glucose levels (Glucometer Elite; Bayer Corporation) of rats were determined and the rats were euthanized with 30% urethane. HbA1c (an estimate of the average level of hyperglycemia over the previous 2−3 months) was measured by a HPLC method as described previously (33). Also at the end of the study, fructosamine (an estimate of the average level of hyperglycemia over the previous 2−3 weeks) (Aeroset System; Abbott Laboratories, Abbott Park, IL) and triglyceride levels were measured (Accutrend GCT meter; Roche Diagnostics, Basel, Switzerland). These experiments conformed to the ARVO Resolution on the Use of Animals in Research.

Tissue Samples

After rats were sacrificed, both eyes were enucleated and one eye from each animal was frozen at −80°C for measurements for MDA and aldose reductase activity. Three eyes from each group were placed in buffered 2.5% glutaraldehyde solution for retinal capillary basement membrane thickness (RCBMT) evaluation and three eyes from each group were placed in 10% neutral formalin for VEGF immunohistochemistry.

Malonyldialdehyde Levels

Malonyldialdehyde (MDA), which is an end product of fatty acid peroxidation, reacts with thiobarbituric acid to form a colored complex that has maximum absorbance at 532 nm (34). To measure MDA levels in retina samples, 0.2 mL of homogenate was suspended in 0.8 mL phosphate-buffered saline (PBS) and 0.025 mL butylated hydroxytoluene as described previously (34). Thirty percent trichloroacetic acid was then added. Tubes were vortexed and allowed to stand in ice for at least 2 h. Tubes were centrifuged at 2000 $\times$ g for 15 min. One mL each of the supernatant was transferred to another tube. To this we added 0.075 mL 0.1 M EDTA and 0.25 mL of 1% TBA in 0.05 N NaOH. Tubes were mixed and kept in a boiling-water bath for 15 min. Absorbance was read at 532 and 600 nm after tubes were cooled to room temperature. Absorbance at 600 nm was subtracted from absorbance at 532 nm. MDA values in nanomoles were determined with the extinction coefficient of MDA-TBA complex at 532 nm $= 1.56 \times 10^5\ \mathrm{cm}^{-1}\ \mathrm{M}^{-1}$.

Aldose Reductase Enzyme (AR) Activity Measurement

After homogenization with a glass homogenizer containing 0.5 mM EDTA, 10 mM of 2-mercaptoethanol and 1 mL of 20 mM PBS, pH 7.0, the tissue samples were centrifuged at 5000 $\times$ g for 5 min. The pellet was placed into PBS and centrifuged once more (10 min at 4°C, 25,000 $\times$ g). In the provided supernatant activity of the enzyme, AR and the quantity of protein was measured.

AR was determined spectrophotometrically according to the method of Das and Skrivastava (35) by monitoring the decrease in NADPH absorption at 340 nm at 37°C using DL-glyceraldehyde as substrate. Unspecific NADPH dehydrogenase activity was recorded for 5 min; then DL-glyceraldehyde was added and the incubation was continued for another 5 min. Values of AR activity given in this study represent the difference between the rate of NADPH oxidation in the presence and absence of substrate.

Measurement of Retinal Capillary Basement Membrane Thickness (RCBMT)

For evaluation of RCBMT, the eyes were removed, slit and fixed by immersion for several days in a solution of 2.5% glutaraldehyde and 6% sucrose buffered to pH 7.2 in 50 mM sodium cacodylate. Two portions of retina, approximately 0.25 $\times$ 0.8 mm and radially oriented, were dissected from the superior temporal quadrant near the optic disc (temporal quadrant was marked by the help of the muscle nictitans found on the nasal side). These were postfixed in 1.0% OsO_4 buffered to pH 7.2 with 150 mM Na/K phosphate buffer and embedded in Epon 812 epoxy resin. Sectioned tissues were stained with aqueous uranyl acetate and lead citrate and were then examined in a Zeiss EM electron microscope.

Electron micrographs were taken of all the perpendicular or near perpendicular cross-sectional cuts of the capillaries

found in the outer plexiform layer of both retinal samples from three of the animals in each experimental group.

Capillaries of other retinal layers were not used for RCBMT evaluation. Using masked procedures, micrographs for analysis were selected by only two criteria: first, that the image represents a cleanly cut, perpendicular cross-section with unambiguous basement membrane borders, and second, that the vessel be of capillary diameter (~5–8 μm outside diameter). The pixel value of the distance between two points on the measurement scales of the photos was measured by the use of the program, Paint Shop Pro-4. Basement membrane thickness value measured as pixel on digital photos was converted to nanometers according to a previously determined equation with the measurement scale on photos. From each eye three capillaries and from each capillary vessel eight sections were measured. Thus, from each group 72 measurements were gathered for statistical evaluation.

Immunohistochemistry for VEGF

Three eyes in each group were fixed in 10% neutral formalin for 72 h. After routine immunohistochemical procedure, 4- to 5-μm sections were cut from the paraffin blocks and mounted on polylysine-coated slides. To localize VEGF immunohistochemically, a monoclonal anti-VEGF antibody (cat #MS-1467-R7, VEGF Ab-7, Clone VG1, Neomarkers, Labvision, Fremont, CA) was used.

Deparaffinized sections were rehydrated. After rehydration, endogenous peroxidase was used to eliminate nonspecific background staining and the sections were incubated in hydrogen peroxide for 10–15 min. When needed, the sections were digested with 0.01% chymotrypsin (Sigma) for 20 min and washed with buffered saline following incubation. Primary VEGF antibody was applied according to the protocol and incubation was done for 30 min. For immunostaining, primary antibody was applied to the sections for their attachment to antigen and later biotinylated secondary antibody was attached to this molecule. Finally, for acquiring a visible product, DAB (3,3′-diamino benzidine) was added to the medium as substrate. VEGF immunoreactivity observed in the cytoplasm of endothelial cells of choroidal capillaries was examined at light microscopic high magnification (×1000), and VEGF immunostaining was assessed by two independent investigators blinded to the group of the sections. Staining intensity was graded qualitatively as weak (corresponding to the level of staining seen in the controls), moderate and intense (corresponding to the level of highest immunoreactivity).

Statistical Analysis

Data are reported as mean ± SEM and analyzed statistically using analysis of variance (ANOVA) followed by post-hoc Bonferroni test for multiple-group comparison; p values <0.05 were considered statistically significant. Similar conclusions were also reached by using nonparametric Kruskal-Wallis test followed by Mann-Whitney U test for multiple-group comparison.

Results

Blood Analysis

STZ administration affected the levels of typical blood parameters characteristic for diabetes, which are also accepted tools in diabetes diagnostics (glucose, glycated hemoglobin, fructosamine), indicating high levels of glycosylation. Blood glucose, HbA1c and fructosamine concentrations were significantly increased in untreated diabetic rats compared to control groups ($p<0.001$) (Figures 1A and 1B). When diabetic animals were treated with each antioxidant alone for 8 months, significant decreases in blood glucose, HbA1c and fructosamine concentrations were observed in diabetic rats. Antioxidant treatment also resulted in a significant decrease in mean plasma triglyceride concentration of diabetic animals. However, the combined treatment with both antioxidants (vitE plus ST) did not induce additional (significant) effect on glycemic control of diabetic rats compared to the treatments of each antioxidant alone (Figures 1A and 1B).

Malonyldialdehyde (MDA) Levels

The retina of untreated diabetic rats had considerably increased MDA levels compared with controls ($p<0.001$). A statistically significant reduction of MDA was found in retina of diabetic rats when the diabetic rats were treated with ST or vitamin E. The combined effect of ST and vitamin E on MDA was greater than that of ST or vitamin E alone, and antioxidant combination achieved a complete amelioration in MDA levels of diabetic retina (Figure 2).

Aldose Reductase Enzyme (AR) Activity

When compared with non-diabetic control group, diabetic rats without antioxidant treatment had significantly higher AR activity ($p<0.001$). ST or vitE treatment resulted in a significant decrease in AR activity in diabetic rats compared to untreated diabetic rats ($p<0.001$). The combination of two antioxidants leads to more amelioration in AR activity of diabetic rats when compared to the effects of each antioxidant treatment alone ($p<0.05$) (Table 1).

Evaluation of Retinal Capillary Basement Membrane Thickness (RCBMT)

Mean RCBMT was significantly increased in untreated diabetic rats when compared with RCBMT value obtained from control rats ($p<0.001$) (Table 1). RCBMT was significantly decreased in diabetic rats receiving either ST or vitE compared to diabetic rats without antioxidant treatment. However, vitE-treated diabetic and ST-treated

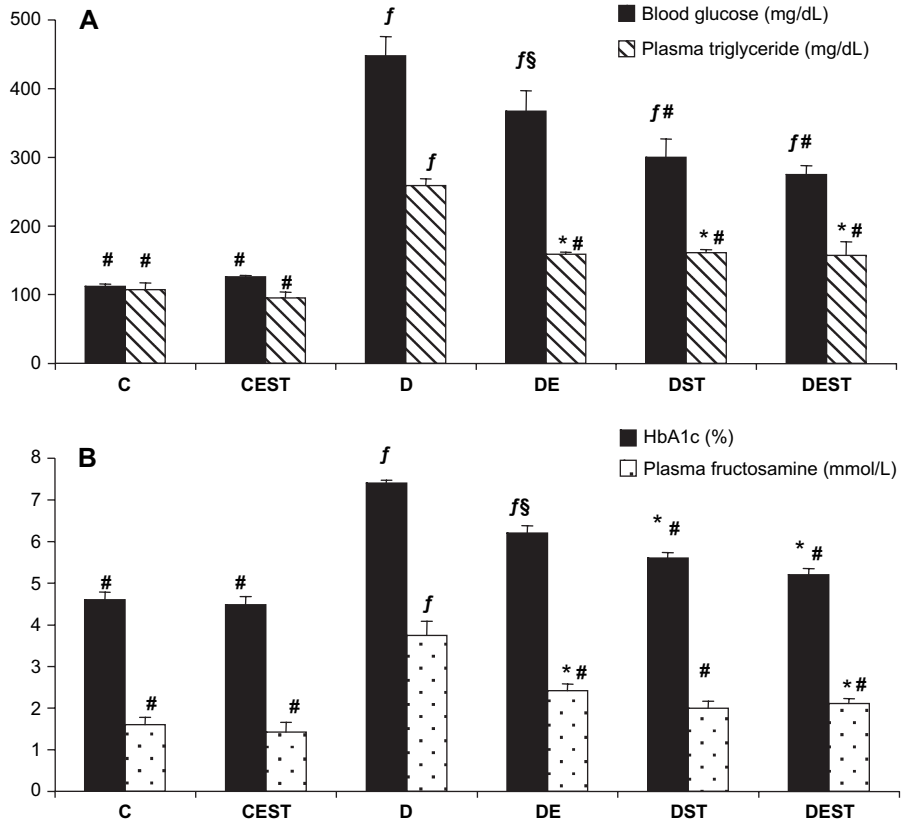

Figure 1. (A) Blood glucose and plasma triglyceride. (B) HbA1c and plasma fructosamine concentrations of rats (mean ± SEM). C, control rats untreated with antioxidants; CEST, control rats treated with antioxidant combination; D, diabetic rats untreated with antioxidants; DE, diabetic rats treated with vitamin E; DST, diabetic rats treated with stobadine; DEST, diabetic rats treated with antioxidant combination (vitamin E plus stobadine). For each group $n = 10$ rats. Those that have statistically significant difference from control rats untreated with antioxidants (*f*): $p < 0.001$, *$p < 0.05$. Those that have statistically significant difference from diabetic rats untreated with antioxidants: #$p < 0.001$, §$p < 0.05$.

diabetic rats still had significantly thicker RCBMT as compared to the non-diabetic control rats. When diabetic rats were treated with antioxidant combination, RCBMT did not demonstrate a statistically significant difference than that of non-diabetic control rats ($p > 0.001$) (Table 1).

Immunohistochemistry for VEGF

Weak VEGF immunoreactivity was exhibited in the ganglion cell layer, inner plexiform layer and inner nuclear layer in retinal sections examined in control groups (Figure 3A). VEGF immunoreactivity was noted as weak in the inner plexiform layer, moderate in the inner nuclear layer and intense in the optic nerve fiber layer in diabetic rats without antioxidant treatment (Figure 3B). Immunostaining in the choroidal capillaries was compared between groups. Intense immunoreactivity was seen in diabetic rats without antioxidant treatment (Figure 4A), whereas moderate immunostaining was observed in diabetic rats with either ST (Figure 4B) or vitE treatment (Figure 4C), and weak immunostaining was noted in diabetic groups with combined ST and vitE treatment (Figure 4D) and in all of the control groups (Figure 4E).

Discussion

Intracellular ROS accumulation has been proposed as the common element linking major signaling pathways implicated in hyperglycemia-induced retinal abnormalities. Supporting the involvement of oxidative modifications in diabetic retinal changes, the beneficial effects of some antioxidant supplements have been demonstrated in different models of diabetic retinal disease (18–28). Oxidative stress, increasing in diabetic retina during hyperglycemia, is linked to augmented retinal basement thickening, which is an early morphological characteristic of the microangiopathy seen in diabetic retinal disease (6,19,36). In accordance with previous observations, our study demonstrated that the increased RCBMT is associated with enhanced retinal oxidative stress (MDA, lipid peroxidation) in diabetic rats. In the present study we observed a 37.99% increase in RCBMT in rats after 8 months diabetes duration. The increase in RCBMT was 12.34% in diabetic rats with ST treatment and 23.07% in diabetic rats with vitE treatment. In the group treated with antioxidant combination, only a 4.38% increase was observed in RCBMT.

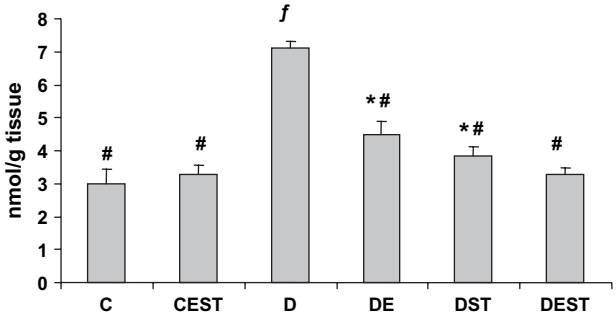

Figure 2. Lipid peroxidation (malondialdehyde, MDA) levels in retina of rats. C, control rats untreated with antioxidants ($n = 8$); CEST, control rats treated with antioxidant combination ($n = 5$); D, diabetic rats untreated with antioxidants ($n = 8$); DE, diabetic rats treated with vitamin E ($n = 7$); DST, diabetic rats treated with stobadine ($n = 9$); DEST, diabetic rats treated with antioxidant combination (vitamin E plus stobadine) ($n = 8$). Those that have statistically significant difference from control rats untreated with antioxidants *f*$p < 0.001$, *$p < 0.05$. Those that have statistically significant difference from diabetic rats untreated with antioxidants: #$p < 0.001$.

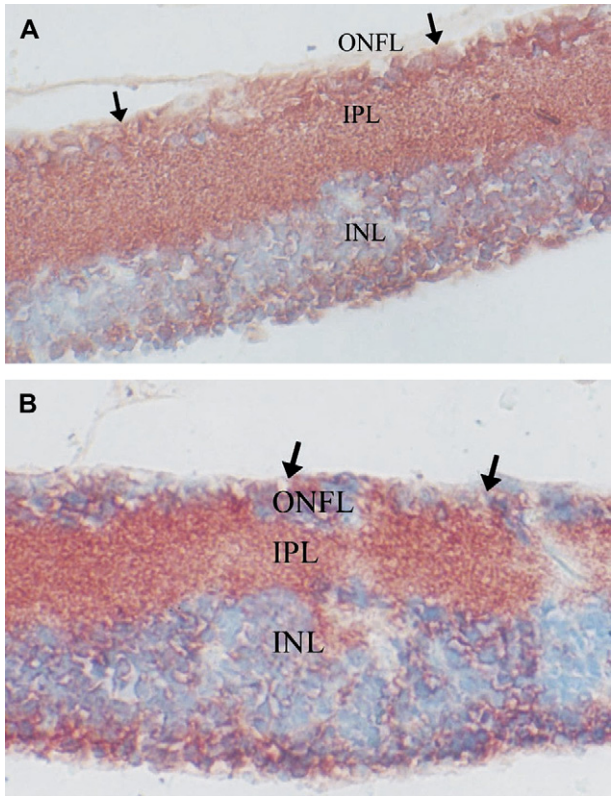

Figure 3. (A) Retinal section of a rat in control group without antioxidant supplementation (×400). Weak VEGF immunoreactivity is observed in the inner plexiform (IPL), inner nuclear (INL) layers. Black arrows show the moderate VEGF immunoreactivity observed in optic nerve fiber layer (ONFL). (B) Retinal section of a diabetic rat without antioxidant supplementation (×400). Moderate VEGF immunoreactivity was observed in the inner plexiform layer (IPL) and inner nuclear (INL) layer, whereas intense immunoreactivity was present in the nerve fiber layer (ONFL). Black arrows show strong VEGF immunoreactivity observed in the ONFL.

As in the case of the present study, we have already demonstrated the recovering effect of each antioxidant compound on typical characteristics of experimental diabetes including blood glucose, HbA1c, plasma fructosamine and triglyceride levels (37,38). These observations even suggest an opinion that the interventions of ST or vitE on metabolic control of diabetic rats are a plausible explanation for their individual beneficial effects on RCBMT in diabetic rats, although it seems to be insufficient because the effects of each antioxidant on the severity of diabetes and metabolic control were only partial and their combined effects were not more robust than their individual effect. However, we found an arrested increase in retinal lipid peroxidation (MDA) and complete amelioration in RCBMT in diabetic rats treated with antioxidant combination, identifying the more important role of increased oxidative stress in the appearance of morphological modifications observed in diabetic retina. Consistent with this, previous studies showed that oxidative modifications in the retina occur early in the course of development of retinopathy in diabetic rats and are not easily reversed by just reinstitution of good glycemic control (3). It has been widely reported that activated polyol (sorbitol) pathway of glucose metabolism is a good reason in the explanation of increased oxidative stress in diabetic rats, which is supported by the counteracting effects of AR inhibitors (11–15). In addition,

Table 1. Aldose reductase enzyme (AR) activity and retinal capillary basement membrane thickness (RCBMT) of rats (mean ± SEM)

	C	CEST	D	DE	DST	DEST
AR activity (μmol/mg protein/hour)	0.37 ± 0.02#	0.34 ± 0.16#	4.22 ± 0.23 *f*	1.94 ± 0.13 *f* #	1.96 ± 0.15 *f* #	0.80 ± 0.06#
RCBMT (nm)	79.2 ± 1.23#	81.27 ± 1.21# (2.62%)	109.29 ± 1.49 *f* (37.99%)	97.47 ± 2.11 *f* § (23.07%)	88.97 ± 1.39* # (12.34%)	82.67 ± 1.52# (4.38%)

C, control rats untreated with antioxidants; CEST, control rats treated with antioxidant combination; D, diabetic rats untreated with antioxidants; DE, diabetic rats treated with vitamin E; DST, diabetic rats treated with stobadine; DEST, diabetic rats treated with antioxidant combination (vitamin E plus stobadine). For RCBMT n = 3 rats, for AR activity n = 10 rats in each group.

Changes in retinal capillary basement membrane thickness (RCBMT) compared to control are expressed in % of the control group (in parantheses) e.g. (2.62%) etc. Those that have statistically significant difference from control rats untreated with antioxidants: (*f*): $p < 0.001$, (*): $p < 0.05$.

Those that have statistically significant difference from diabetic rats untreated with antioxidants: (#): $p < 0.001$, (§): $p < 0.05$.

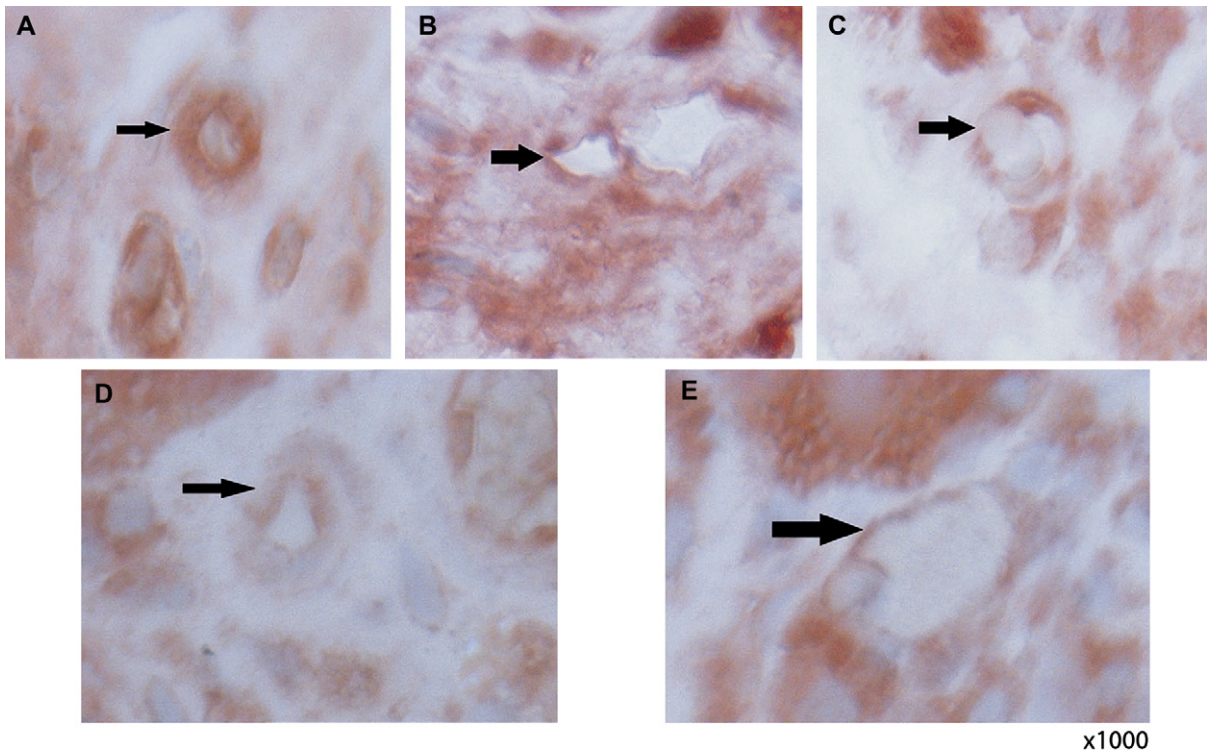

Figure 4. Photomicrographs demonstrating VEGF immunoreactivity in the choroidal vasculature. VEGF immunostaining was observed as intense in the capillary walls (black arrow) of choroidal layer in diabetic rats without treatment (A). On the other hand, VEGF immunostaining of the capillary walls (black arrow) of choroidal layer in diabetic rats with ST treatment (B) and that (black arrow) of rats with vitamin E treatment (C) was moderate; whereas immunostaining of the capillary walls (black arrow) of choroidal layer in diabetic rats with combined ST and vitamin E treatment (D) and that (black arrow) of the control group rats was weak (E).

hyperglycemia-mediated increased AR protein and mRNA has been found to inhibit by vitamin E (10). In accordance with previous observations, we determined an activated AR in retina of untreated diabetic rats, which was greatly inhibited by the treatments with both antioxidants.

On the other hand, an important role for enhanced vascular endothelial growth factor (VEGF) expression in the origin of the retinal microcirculatory dysfunction seen in diabetes, characterized by thickened basement membranes, pericyte loss, capillary dropout, and increased circulation time, has been well documented. It has also been reported that oxidative stress is involved in the upregulation of retinal VEGF during early diabetes, and the increased VEGF immunoreactivity is inhibited by the inhibitors of AR, AGEs, or antioxidants in diabetic rats (13,39−41). In accordance with this, we observed intense VEGF immunostaining in the retinal optic nerve fiber layer and also the choroidal capillaries in untreated diabetic rats. In the choroidal capillaries of diabetic rats treated with antioxidants, VEGF immunostaining is largely decreased (with combined antioxidant supplement, even almost to the level of that of the control group).

The effectiveness of different antioxidant compounds, vitamins, minerals or their combinations in the development of diabetic retinal pathology and metabolic abnormalities has previously been widely reported (18−24,40,41). In this context, we believe that the essential ability of vitamin E and ST combination on the suppression of retinal pathology, without action of total glycemic/lipidemic control in diabetic rats, is likely due to the capacity of their combination to decrease retinal modifications caused by harmonic connections of diabetes-induced peroxidative, carbonyl and nitrosative stresses or other characteristics of the pathologies of diabetic retinopathy. We previously showed that treatment with vitamin E (400−500 IU/kg/day, orally) or ST (24.7 mg/kg/day, orally) significantly inhibits lipid peroxidation and free radical generation. In addition, it ameliorates enzymes with roles in the pentose phosphate pathway, glutathione-dependent, and other antioxidant defense mechanisms in blood and tissues such as kidney, aorta, brain, heart or liver of diabetic rats (31,32,38,42). Recently, we also found that the visual cataract score is significantly decreased in the diabetic model of cataractogenesis by ST treatment, whereas vitamin E had no significant effect (43). However, ST and Trolox (a synthetic vitE analogue) significantly inhibited the aggregation and cross-linking of eye lens proteins in a model of peroxyl radical-mediated cataractogenesis (44). Together the effects of ST on diabetic retinal changes may not be mediated simply by its ability to attenuate lipoxidation reactions and the production of toxic aldehydes and other properties of ST such as membrane

stabilization, prevention of calcium accumulation (31) and mitochondrial energy ameliorating effects (29). These may have important roles in the mechanisms of its beneficial effects on diabetic retina.

On the other hand, studies have demonstrated depletion of vitamin E levels in the diabetic state, promoting tissue damage (45). Although there is absence of significant changes mediated by diabetic retinopathy in vitamin E-deprived rats (36), in our study, supplementation of vitamin E might counteract the decreased intra- and extracellular vitE levels associated with diabetic retinal abnormalities. As indicated previously, it is likely that an antioxidant would not exert its effect greatly by itself *in vivo* but would act in combination with other antioxidants present. Such interactions may be effective in the *in vivo* production of final antioxidant capacity. Indeed, these investigators demonstrated the ability of a water-soluble analogue of α-tocopherol, Trolox, to recycle ST from its one electron oxidation product yielding a corresponding Trolox radical (46). Moreover, it has been demonstrated that the antioxidant potency of vitamin E and ST are increased *in vivo* (47). Thus, the interactions of vitamin E and ST have an important role in the production of their pronounced effect on retinal alterations in diabetic rats.

In conclusion, the present study demonstrated that individual supplementation of ST and vitE was effective in reducing RCBMT in diabetic rats. This finding is associated with inhibition of retinal lipid peroxidation. Our findings are also consistent with downstream localization of diabetes-induced oxidative stress consequent to increased AR activity which, therefore, renders VEGF upregulation preventable by both antioxidants. The ability of the combination antioxidant treatment to arrest RCBMT and retinal lipid peroxidation in the presence of poor glycemic control might advocate the key role of direct oxidative damage in retinal abnormalities associated with diabetic retinopathy. Due to the fact that diabetic retinopathy appears to be a multifactorial process, our findings support the rationale for evaluating potent antioxidants such as ST and vitE, dietary antioxidant mixtures and antioxidants containing combination therapies to prevent the onset or progression of diabetic retinopathy.

Acknowledgments

This study was supported by the Turkish Scientific and Research Council (TUBITAK), SBAG-2397 and SBAG-SLOVAK-2 (103S180). We also thank Dr. Figen Aktan from the Department of Biochemistry, Faculty of Pharmacy, Ankara University for her kind help.

References

1. Merimee TJ. Diabetic retinopathy: a synthesis of perspectives. N Engl J Med 1990;322:978–983.
2. Aiello LP, Gardner TW, King GL, Blankenship G, Cavallerano JD, Ferris FL 3rd, et al. Diabetic retinopathy (technical review). Diabetes Care 1998;21:143–156.
3. Kowluru RA. Effect of reinstitution of good glycemic control on retinal oxidative stress and nitrative stress in diabetic rats. Diabetes 2003; 52:818–823.
4. Comer GM, Ciulla TA. Pharmacotherapy for diabetic retinopathy. Curr Opin Ophthalmol 2004;15:508–518.
5. Armstrong D, Abdella N, Salman A, Miller N, Rahman EA, Bojancyzk M. Relationship of lipid peroxides to diabetic complications. J Diabetes Complications 1992;6:116–122.
6. Baynes JW. Role of oxidative stress in development of complications in diabetes. Diabetes 1991;40:405–412.
7. Caldwell RB, Bartoli M, Behzadian MA, El-Remessy AE, Al-Shabrawey M, Platt DH, et al. Vascular endothelial growth factor and diabetic retinopathy: role of oxidative stress. Curr Drug Targets 2005;6:511–524.
8. Chung SS, Chung SK. Aldose reductase in diabetic microvascular complications. Curr Drug Targets 2005;6:475–486.
9. Curtis TM, Scholfield CN. The role of lipids and protein kinase Cs in the pathogenesis of diabetic retinopathy. Diabetes Metab Res Rev 2004;20:28–43.
10. Stitt AW, Curtis TM. Advanced glycation and retinal pathology during diabetes. Pharmacol Rep 2005;57(suppl):156–168.
11. El-Remessy AB, Abou-Mohamed G, Caldwell RW, Caldwell RB. High glucose-induced tyrosine nitration in endothelial cells: role of eNOS uncoupling and aldose reductase activation. Invest Ophthalmol Vis Sci 2003;44:3135–3143.
12. Kato N, Yashima S, Suzuki T, Nakayama Y, Jomori T. Long-term treatment with fidarestat suppresses the development of diabetic retinopathy in STZ-induced diabetic rats. J Diabetes Complications 2003;17:374–379.
13. Obrosova IG, Minchenko AG, Vasupuram R, White L, Abatan OI, Kumagai AK, et al. Aldose reductase inhibitor fidarestat prevents retinal oxidative stress and vascular endothelial growth factor overexpression in streptozotocin-diabetic rats. Diabetes 2003;52:864–871.
14. Obrosova IG, Pacher P, Szabo C, Zsengeller Z, Hirooka H, Stevens MJ, et al. Aldose reductase inhibition counteracts oxidative-nitrosative stress and poly(ADP-ribose) polymerase activation in tissue sites for diabetes complications. Diabetes 2005;54:234–242.
15. Cheung AK, Fung MK, Lo AC, Lam TT, So KF, Chung SS, et al. Aldose reductase deficiency prevents diabetes-induced blood-retinal barrier breakdown, apoptosis, and glial reactivation in the retina of db/db mice. Diabetes 2005;54:3119–3125.
16. Grant MB, Afzal A, Spoerri P, Pan H, Shaw LC, Mames RN. The role of growth factors in the pathogenesis of diabetic retinopathy. Expert Opin Investig Drugs 2004;13:1275–1293.
17. Aiello LP. Clinical implications of vascular growth factors in proliferative retinopathies. Curr Opin Ophthalmol 1997;8:19–31.
18. Mayer-Davis EJ, Bell RA, Reboussin BA, Rushing J, Marshall JA, Hamman RF. Antioxidant nutrient intake and diabetic retinopathy: the San Luis Valley Diabetes Study. Ophthalmology 1998;105: 2264–2270.
19. Kowluru RA, Tang J, Kern TS. Abnormalities of retinal metabolism in diabetes and experimental galactosemia. VII. Effect of long-term administration of antioxidants on the development of retinopathy. Diabetes 2001;50:1938–1942.
20. Kowluru RA, Koppolu P. Diabetes-induced activation of caspase-3 in retina: effect of antioxidant therapy. Free Radic Res 2002;36: 993–999.
21. Kowluru RA, Koppolu P, Chakrabarti S, Chen S. Diabetes-induced activation of nuclear transcriptional factor in the retina, and its inhibition by antioxidants. Free Radic Res 2003;37:1169–1180.
22. Di Leo MA, Ghirlanda G, Gentiloni Silveri N, Giardina B, Franconi F, Santini SA. Potential therapeutic effect of antioxidants in experimental

diabetic retina: a comparison between chronic taurine and vitamin E plus selenium supplementations. Free Radic Res 2003;37:323−330.
23. Lin J, Bierhaus A, Bugert P, Dietrich N, Feng Y, Vom Hagen F, et al. Effect of R-(+)-alpha-lipoic acid on experimental diabetic retinopathy. Diabetologia 2006;49:1089−1096.
24. Yatoh S, Mizutani M, Yokoo T, Kozawa T, Sone H, Toyoshima H, et al. Antioxidants and an inhibitor of advanced glycation ameliorate death of retinal microvascular cells in diabetic retinopathy. Diabetes Metab Res Rev 2006;22:38−45.
25. Hoffmann J, Alt A, Lin J, Lochnit G, Schubert U, Schleicher E, et al. Tenilsetam prevents early diabetic retinopathy without correcting pericyte loss. Thromb Haemost 2006;95:689−695.
26. SanGiovanni JP, Chew EY. The role of omega-3 long-chain polyunsaturated fatty acids in health and disease of the retina. Prog Retin Eye Res 2005;24:87−138.
27. Yamagishi SI, Matsui T, Nakamura K, Yoshida T, Takeuchi M, Inoue H, et al. Pigment-epithelium-derived factor suppresses expression of receptor for advanced glycation end products in the eye of diabetic rats. Ophthalmic Res 2007;39:92−97.
28. Garay RP, Hannaert P, Chiavaroli C. Calcium dobesilate in the treatment of diabetic retinopathy. Treat Endocrinol 2005;4:221−322.
29. Horakova L, Stolc S. Antioxidant and pharmacodynamic effects of pyridoindole stobadine. Gen Pharmacol 1998;30:627−638.
30. Stolc S, Vlkolinsky R, Pavlasek J. Neuroprotection by the pyridoindole stobadine: a minireview. Brain Res Bull 1997;42:335−340.
31. Pekiner B, Ulusu NN, Das-Evcimen N, Sahilli M, Aktan F, Stefek M, et al. Antioxidants in Diabetes-Induced Complications Study Group. In vivo treatment with stobadine prevents lipid peroxidation, protein glycation and calcium overload but does not ameliorate Ca^{+2}-ATPase activity in heart and liver of streptozotocin-diabetic rats: comparison with vitamin E. Biochim Biophys Acta 2002;1588:71−78.
32. Ulusu NN, Sahilli M, Avci A, Canbolat O, Ozansoy G, Ari N, et al. Pentose phosphate pathway, glutathione-dependent enzymes and antioxidant defense during oxidative stress in diabetic rodent brain and peripheral organs: effects of stobadine and vitamin E. Neurochem Res 2003;28:815−823.
33. Ito M, Miura J, Ito M, Umesato F, Yasuda K, Takata Y. Correlative retention time peak identification method for glycated haemoglobin in high-performance liquid chromatography. J Chromatogr A 1994;661: 143−151.
34. Jain SK, Levine SN. Elevated lipid peroxidation and vitamin E-quinone levels in heart ventricles of streptozotocin-treated diabetic rats. Free Radic Biol Med 1995;18:337−341.
35. Das B, Skrivastava SK. Purification and properties of aldose reductase and aldehyde reductase II from human erythrocyte. Arch Biochem Biophys 1985;238:670−679.
36. Robison WG, Jacot JL, Katz ML, Glover JP. Retinal vascular changes induced by the oxidative stress of alpha-tocopherol deficiency contrasted with diabetic microangiopathy. J Ocul Pharmacol Ther 2000; 16:109−120.
37. Gunes A, Ceylan A, Sarioglu Y, Stefek M, Bauer V, Karasu C. The Antioxidants in Diabetes-Induced Complications (ADIC) Study Group. Reactive oxygen species mediate abnormal contractile response to sympathetic nerve stimulation and noradrenaline in the vas deferens of chronically diabetic rats: effects of in vivo treatment with antioxidants. Fundam Clin Pharmacol 2005;19:73−79.
38. Demiryurek AT, Karasu C, Stefek M, Stolc S. Effect of stobadine on leukocyte free radical generation in streptozotocin-diabetic rats: comparison with vitamin E. Pharmacology 2004;70:1−4.
39. Kuroki M, Voest EE, Amano S, Beerepoot LV, Takashima S, Tolentino M, et al. Reactive oxygen intermediates increase vascular endothelial factor expression in vitro and in vivo. J Clin Invest 1996;98:1667−1675.
40. Frank RN, Amin R, Kennedy A, Hohman TC. An aldose reductase inhibitor and aminoguanidine prevent VEGF expression in rats with long-term galactosemia. Arch Ophthalmol 1997;115:1036−1047.
41. Obrosova IG, Minchenko AG, Marinescu V, Fathallah L, Kennedy A, Stockert CM, et al. Antioxidants attenuate early up regulation of retinal vascular endothelial growth factor in streptozotocin-diabetic rats. Diabetologia 2001;44:1102−1110.
42. Karasu C, Ozansoy G, Bozkurt O, Erdogan D, Omeroglu S. Antioxidant and triglyceride-lowering effects of vitamin E associated with the prevention of abnormalities in the reactivity and morphology of aorta from streptozotocin-diabetic rats. Antioxidants in Diabetes-Induced Complications (ADIC) Study Group. Metabolism 1997;46: 872−879.
43. Kyselova Z, Gajdosik A, Gajdosikova A, Ulicna O, Mihalova D, Karasu C, et al. Effect of the pyridoindole antioxidant stobadine on development of experimental diabetic cataract and on lens protein oxidation in rats: comparison with vitamin E and BHT. Mol Vis 2005;11: 56−65.
44. Stefek M, Kyselova Z, Rackova L, Krizanova L. Oxidative modification of rat eye lens proteins by peroxyl radicals in vitro: protection by the chain-breaking antioxidants stobadine and Trolox. Biochim Biophys Acta 2005;741:183−190.
45. Naziroglu M, Cay M. Protective role of intraperitoneally administered vitamin E and selenium on the antioxidative defense mechanisms in rats with diabetes induced by streptozotocin. Biol Trace Elem Res 2001;79:149−159.
46. Steenken S, Sundquist AR, Jovanovic SV, Crockett R, Sies H. Antioxidant activity of the pyridoindole stobadine. Pulse radiolytic characterization of one-electron-oxidized stobadine and quenching of singlet molecular oxygen. Chem Res Toxicol 1992;5:355−360.
47. Kagan VE, Tsuchiya M, Serbinova E, Packer L, Sies H. Interaction of the pyridoindole stobadine with peroxyl, superoxide and chromanoxyl radicals. Biochem Pharmacol 1992;45:393−400.