

Aldose Reductase Inhibitor, Epalrestat, Reduces Lipid Hydroperoxides in Type 2 Diabetes

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Abstract. The increased flux of polyol pathway induced by hyperglycemia is implicated in the pathogenesis of various complications associated with diabetic, which results in increased oxidative stress. Because oxidative stress causes tissue damage in patients with diabetes, searching for an effective strategy to reduce oxidative stress in clinical setting is important in order to prevent diabetic complications. The aim of this study was to evaluate the effects of aldose reductase inhibition on oxidative stress in patients with type 2 diabetes mellitus. The subjects of this study were 21 patients with type 2 diabetes. We compared the levels of various oxidative stress markers and antioxidants including plasma thiobarbituric acid-reactive substances, malondialdehyde-modified low-density lipoprotein, vitamin E, β -carotene and lipid hydroperoxides in erythrocytes at baseline with those measured after a 3-month course of epalrestat (150 mg/day), an aldose reductase inhibitor. While administration of epalrestat did not result in significant changes in plasma thiobarbituric acid-reactive substances, malondialdehyde-modified low-density lipoprotein, vitamin E, or β -carotene, it significantly reduced lipid hydroperoxides in erythrocytes. Given the importance of measuring lipid hydroperoxides in erythrocytes as an index of oxidative stress, these results highlight the potential usefulness of epalrestat in reducing oxidative stress in type 2 diabetes mellitus.

Key words: Oxidative stress, Lipid hydroperoxides, Diphenyl-1-pyrenylphosphine (DPPP), Diabetes mellitus, Aldose reductase inhibitor, Epalrestat

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DIABETIC patients are exposed to increased oxidative stress. Oxidative stress impairs various cellular functions and plays important roles in the pathophysiology of various diseases. The progression of diabetes-related complications seems to be mediated at least in part by the increased oxidative stress. Glucose auto-oxidation [1], increased production of glycation end-products (AGE) [2, 3], activation of protein kinase C (PKC) [4], activation of the hexosamine pathway [5], overproduction of reactive oxygen species (ROS) by the mitochondria [6], and the increased flux of polyol pathway [7, 8] are all potential mechanisms of hyperglycemia-induced oxidative stress.

The polyol pathway, a glucose shunt that channels excess glucose to form fructose, encompasses two enzymes. The first enzyme, aldose reductase (AR), reduces glucose to sorbitol with the aid of its co-factor reduced nicotinamide adenine dinucleotide phosphate (NADPH). The second enzyme, sorbitol dehydrogenase (SDH), with its co-factor nicotinamide adenine dinucleotide (NAD), converts sorbitol to fructose. This pathway bypasses the glycolytic pathway, but the flux via the polyol pathway is very small under normoglycemia because the K_m value of AR for glucose is much higher than that of hexokinase that converts glucose to glucose-6-phosphate. However, when glu-

Table 1. Clinical characteristics of study subjects

	Epalrestat group	Control group	Healthy group
N	21	11	15
Age (years)	62.5 ± 9.4	66.9 ± 8.7	32.6 ± 4.8
Sex (male/female)	13/8	8/3	11/4
BMI (kg/m ²)	22.5 ± 3.8	24.9 ± 4.1	NA
Duration of diabetes (years)	15.5 ± 14.3	17.8 ± 12.2	NA
Complications (n) (non/retinopathy/nephropathy)	6/8/10	3/7/7	NA
Therapy (n) (diet/OHA/insulin)	5/12/4	2/8/1	NA

Data are expressed as mean ± SD. BMI: Body mass index, NA: not applicable, OHA: oral hypoglycemic agents.

cose level is elevated, the flux of polyol pathway is enhanced, which results in accumulation of sorbitol [9]. Since sorbitol poorly penetrates the cell membrane, its subsequent accumulation modifies the process of osmoregulation and may contribute on the progression of diabetic complications, such as cataract, neuropathy, and nephropathy [10–12]. This is one of the important mechanisms through which the polyol pathway can induce cell damage.

On the other, the polyol pathway does not only generate osmotic stress but also hyperglycemic oxidative stress. While there are several mechanisms through which the polyol pathway can increase oxidative stress, the enhanced polyol pathway decreases NADPH/nicotinamide adenine dinucleotide phosphate (NADP) ratio [7, 13], thus increasing the ratio of glutathione (γ -glutamylcysteinyl glycine [GSH])/glutathione disulfide (GSSG). This change results in reduced capacity

for oxidation defense. Numerous experimental studies demonstrated the effectiveness of aldose reductase inhibitors (ARI) in animal models [14, 15], but a relatively small number of clinical studies of ARI with regard to its effect on oxidative stress has been reported [13, 16, 17]. Furthermore, there is little or no information on whether treatment with ARI can suppress the formation of lipid hydroperoxide in tissues of diabetic patients.

The aim of the present study was to determine the antioxidant effects of epalrestat in patients with type 2 diabetes using various oxidative markers including lipid hydroperoxides in erythrocytes.

Materials and Methods

Chemicals

Diphenyl-1-pyrenylphosphine (DPPP) was purchased from Dojindo Laboratories (Kumamoto, Japan). Other reagents and solvents used were super-pure grade reagents purchased from Wako Pure Chemical Co. (Osaka, Japan).

Subjects

To evaluate the effects of epalrestat (150 mg/day), the patients with type 2 diabetes mellitus who visited Juntendo University Hospital (Tokyo, Japan) from April 2006 to August 2006 were asked to participate in this study. The clinical characteristics of the study subjects are listed in Table 1. The diagnosis of type 2 diabetes was based on the current WHO criteria. Patients with anemia, diabetic microangiopathy, severe renal or hepatic disease, overt cardiovascular disease, and malignancy were excluded. Furthermore, patients

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Abbreviations: 8-epiPGF_{2 α} , 8-epi-prostaglandinF_{2 α} ; AGE, advance glycation endproducts; AR, aldose reductase; ARI, aldose reductase inhibitor; BHT, butylated hydroxytoluene; CML, N-(carboxymethyl) lysine; DPPP, diphenyl-1-pyrenylphosphine; DPPP=O, diphenyl-1-pyrenylphosphine oxide; ELISA, enzyme-linked immunosorbent assay; GSH, reduction of reduced glutathione (γ -glutamylcysteinyl glycine); GSSG, glutathione disulfide; HDL, high density lipoprotein; HPLC, high performance liquid chromatography; LDL, low density lipoprotein; MDA-LDL, malondialdehyde-modified low density lipoprotein; NAD, nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NADP, nicotinamide adenine dinucleotide phosphate; PKC, protein kinase C; ROS, reactive oxygen species; SDH, sorbitol dehydrogenase; TBARS, thiobarbituric acid reactive substances

with more than 2% variation in HbA_{1c} value within 6 months were excluded. None of the subjects had taken vitamin C, vitamin E, or probucol, which are known antioxidant compounds. As a control, we recruited healthy volunteers. The hospital ethics committee approved this study protocol and informed consent was obtained from each subject.

Study protocol

The effect of 3-month course of epalrestat was evaluated in 21 type 2 diabetic patients. Fasting blood samples were obtained at baseline and 12 weeks after administration of epalrestat (150 mg/day) in the treated group. As a control, we obtained fasting blood sample of type 2 diabetic patients without the treatment of epalrestat at 12 week intervals. Each patient was reviewed once a month for evaluation of general health and compliance with medication. The doses of every medication including anti-diabetic drugs were not changed during the study. Also, we obtained blood sample from healthy volunteers.

Lipid extraction

Approximately 5 ml blood was drawn into EDTA-2Na test tube and centrifuged at $1000 \times g$ for 15 min at 4°C. After separation of plasma and removal of the buffy coat, the erythrocytes were washed three times with isotonic saline. The obtained packed erythrocytes were stored at -70°C until use. In the next step, 100 µL of packed erythrocytes in 1 ml of 5 mM ammonium-acetate buffer (pH 7.4) was centrifuged at $40,000 \times g$ for 15 min and the supernatant was removed. The procedure was repeated three times for preparation of erythrocyte ghost. Then, lipid was extracted from the white ghost by the method of Bligh and Dyer [18] with minor modifications. Special care was taken during sample processing to avoid artifactual oxidation (dim light was used throughout the analysis, contact with air was minimized, and all samples were stored under argon). In brief, the sample (resulting white ghost) was mixed with 0.7 ml water (containing acetic acid with final concentration of 0.1 M), 2 ml methanol [containing 0.003% butylated hydroxytoluene (BHT)] and 1 ml chloroform. After 30-min incubation at room temperature under constant stirring, 1 ml chloroform and 1 ml water were added. The samples were mixed, and then centrifuged at $1500 \times g$

for 15 min. After removal of the supernatant, the resultant chloroform layer was collected and evaporated under reduced pressure at 20°C. The residue was dissolved in 1 ml of mixture of methanol and chloroform (1 : 2), which contained 0.003% BHT, and stored at -70°C until measurement.

Determination of total lipid hydroperoxides

DPPP was purchased from Dojindo Laboratories (Kumamoto, Japan). To a screw-cap test tube, we added 100 µl aliquot of lipid solution and 50 µl of DPPP solution [1 mg/10 ml in methanol-chloroform (1 : 1)]. The tube was placed in a dry bath at 60°C for 60 min in the dark. After cooling on an ice bath, 3 ml of methanol was added to the mixture. After shaking, the fluorescence intensity was measured at 380 nm (excitation at 352 nm) using a fluorometer (model F-4500 fluorescence spectrophotometer, Hitachi, Tokyo) and the intensity of fluorescence per lipid weight was calculated. To confirm reproducibility, measurements were repeated four times for both plasma and erythrocytes obtained from the same donor at different times. The distribution of lipid hydroperoxides concentrations showed satisfactory and no substantial change [19].

Measurement of malonaldehyde-modified low-density lipoprotein (MDA-LDL), thiobarbituric acid-reactive substances (TBARS), vitamin E, β-carotene and other laboratory assays

Plasma MDA-LDL levels were measured by enzyme-linked immunosorbent assay (ELISA) based on the principle reported previously [20]. To measure TBARS, a test kit (Wako, Osaka, Japan) was used according to the method described previously [21]. Plasma vitamin E and β-carotene were measured by high performance liquid chromatography (HPLC). Plasma glucose concentrations were determined by the glucose oxidase method (Kainos, Tokyo). Glycosylated hemoglobin (HbA_{1c}) was measured by HPLC (normal range, 4.3–5.8%, TOHSOH, Tokyo). Total-cholesterol, HDL-cholesterol and triglycerides levels were measured using standard enzymatic methods (Kainos) and LDL-cholesterol values were calculated using Friedewald's formula. These assays were performed by hospital personnel in the Department of Clinical Chemistry except for MDA-LDL, TBARS, vitamin E and β-

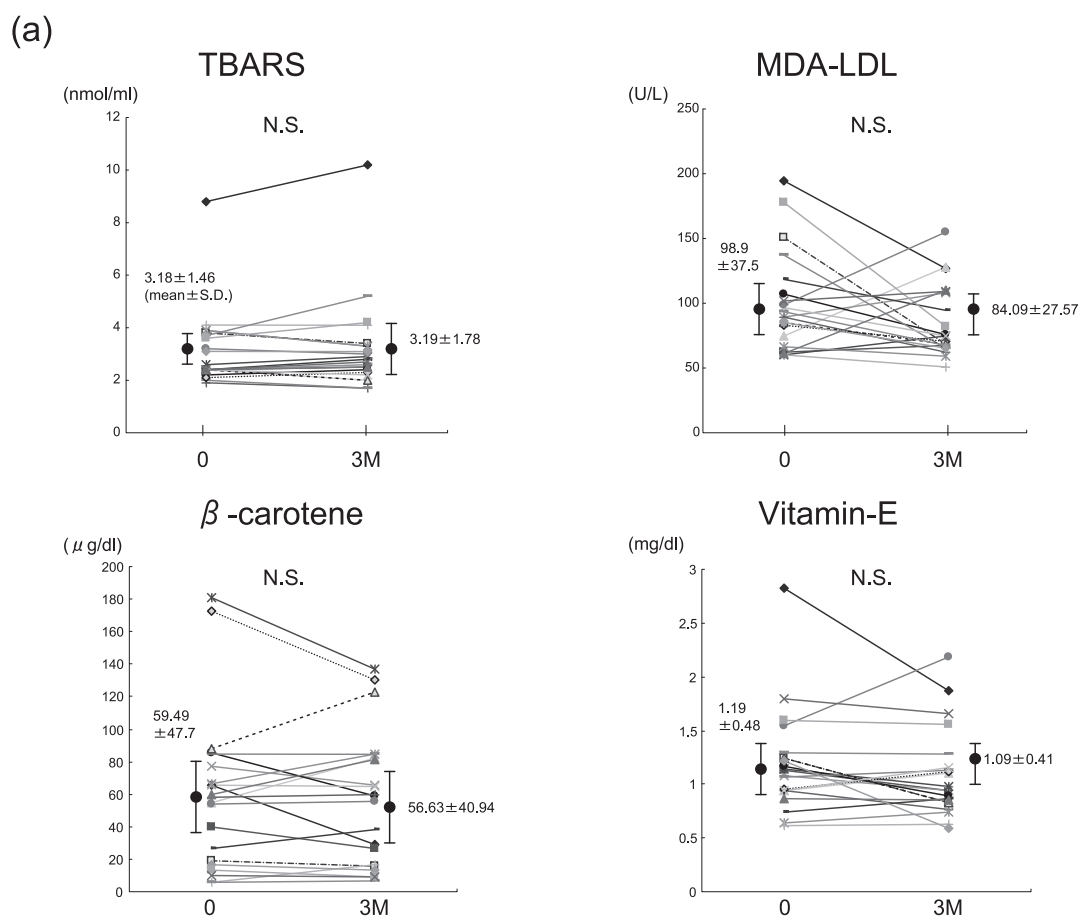


Fig. 1. Effects of treatment with epalrestat on four serum oxidative stress markers. The graph shows data of individual patients with diabetes and the mean \pm SD values with the treatment with epalrestat (a) and control (b). 3M: at the end of the 3-month treatment course.

carotene, which were measured in a commercial laboratory (SRL, Tokyo).

Statistical analysis

Values were presented as mean \pm SD. The Wilcoxon t-test was used to compare differences between baseline and follow-up measures within groups. Significance was defined as $P < 0.05$.

Results

Clinical characteristics of study subjects were shown in Table 1. Table 2 shows the data of plasma glucose, HbA_{1c}, total-cholesterol, HDL-cholesterol, LDL-cholesterol, and triglyceride at baseline and 3 months after the beginning of treatment with epalrestat. Epalrestat

had no effect on any of the parameters tested shown in Table 2. With regard to basal lipid hydroperoxides level, the values in epalrestat and control group was significantly higher than that of healthy group (epalrestat group: 6.19 ± 2.68 , control group: 7.17 ± 5.15 , healthy group: 1.27 ± 0.29). With regard to changes of various serum oxidative stress markers, epalrestat had no effect on any of the markers tested similar to control group (Fig. 1). On the other hand, in contrast to control group, epalrestat significantly reduced lipid hydroperoxides level (baseline: 6.19 ± 2.68 , follow-up: 5.25 ± 2.36 , $p < 0.05$, Fig. 2).

Discussion

To the best of our knowledge, this is the first report that investigated the effect of ARI on lipid peroxida-

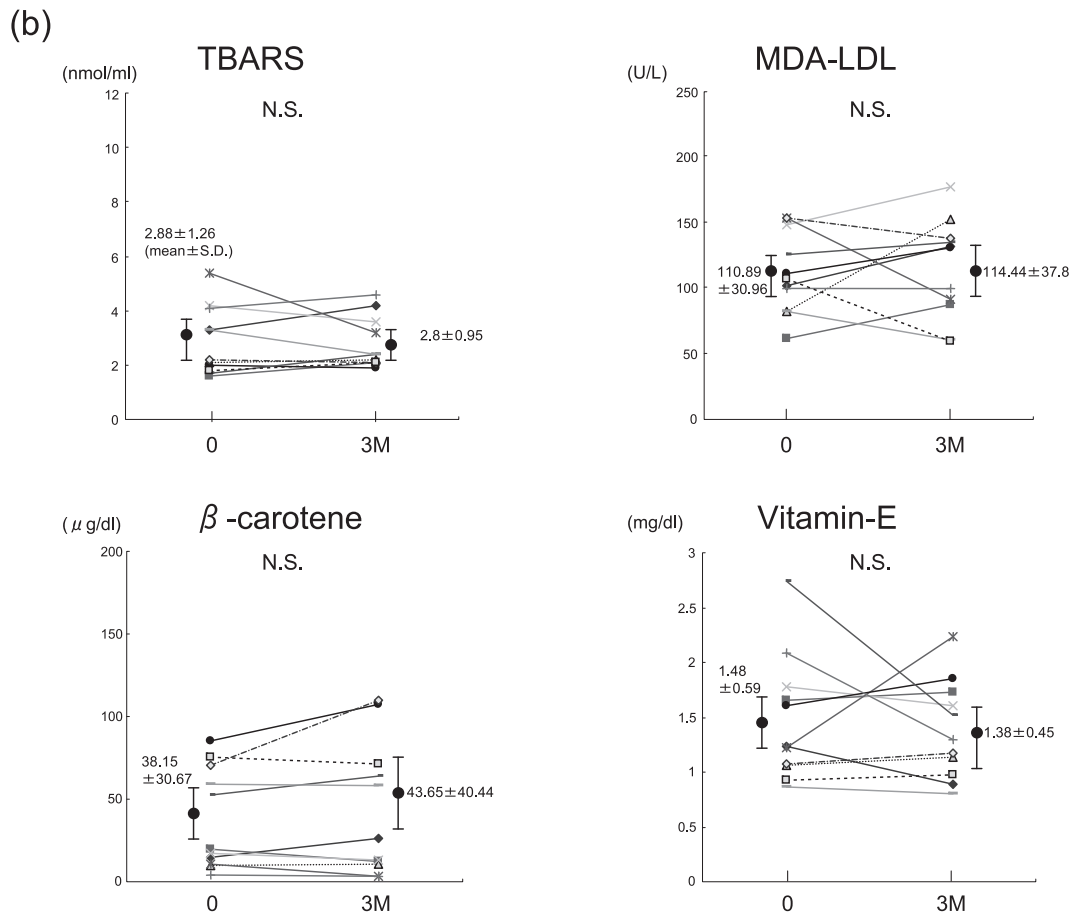


Fig. 1.

Table 2. Laboratory data of each group

	Epalrestat group		Control group		Healthy group
	Baseline	End of the study	Baseline	End of the study	Baseline
Fasting blood sugar (mg/dl)	121.3 \pm 22	132.3 \pm 42.3	131 \pm 31.4	144.7 \pm 29.3	92 \pm 6.6
HbA1c (%)	6.10 \pm 0.8	6.11 \pm 0.72	6.97 \pm 1.0	6.85 \pm 0.95	4.7 \pm 0.2
Total cholesterol (mg/dl)	192.4 \pm 3.2	189.6 \pm 28.6	194.3 \pm 26.5	202.2 \pm 29.8	184.8 \pm 27.5
High-density lipoprotein (mg/dl)	55.3 \pm 13.5	55.1 \pm 14.4	49.3 \pm 9.7	48.2 \pm 9.2	64.8 \pm 12.4
Low-density lipoprotein (mg/dl)	108.6 \pm 27.4	104.5 \pm 25.0	114.3 \pm 21.8	122.8 \pm 27.1	102.1 \pm 23.1
Triglyceride (mg/dl)	137 \pm 113	135 \pm 114	163 \pm 139	154 \pm 85	107.7 \pm 64.4

Data are expressed as mean \pm SD.

tion level in human tissue. Our study showed that lipid hydroperoxides levels in erythrocytes of diabetic patients were significantly reduced following treatment with epalrestat.

In general, it is difficult to evaluate the oxidative stress in human precisely. Diabetic patients are known to be exposed to increased oxidative stress. We found higher lipid hydroperoxides level in diabetic patients

than healthy volunteers. While there are several potential factors affecting lipid hydroperoxides level, these data suggests that glycemia level might be one of such factors.

There are several potential explanations for polyol pathway-induced increase in oxidative stress. Hyperglycemia activates the polyol pathway, and reduction of glucose to sorbitol through the AR may lead to

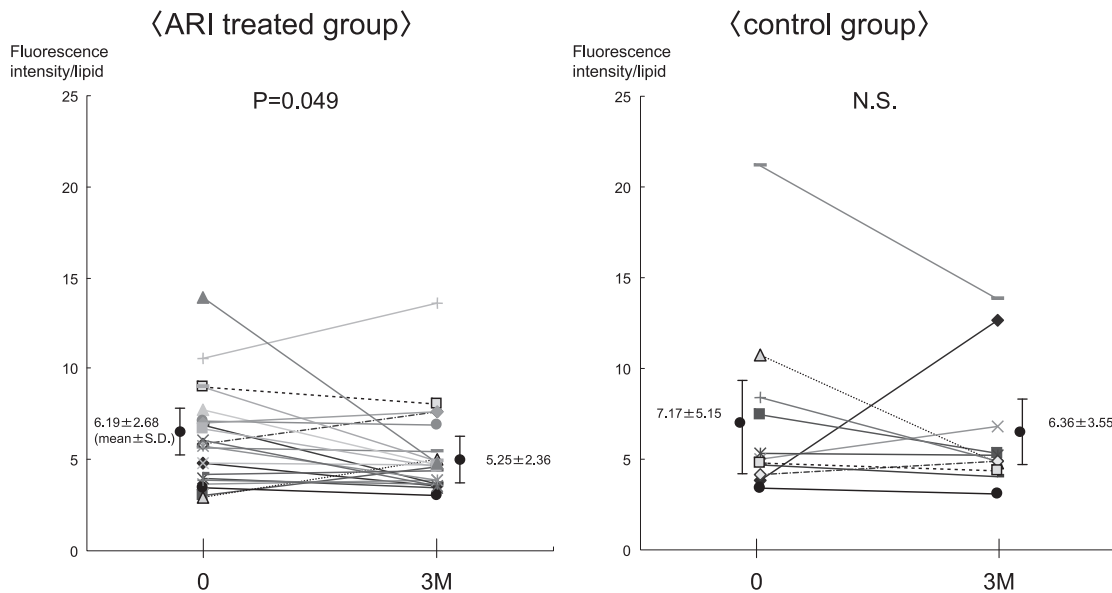


Fig. 2. Lipid hydroperoxides levels before (0) and after a 3-month course of epalrestat treatment (3 M). The graph shows data of individual patients with diabetes and the mean \pm SD values.

NADPH consumption [22]. Because NADPH is used in several critical reductive metabolic steps, such as detoxification of ROS and hydroperoxide, a large drain on the NADPH pool could compromise the ability of the cell to protect itself from oxidative stress. NADPH is also required for glutathione reductase to regenerate GSH, and glutathione peroxidase reduces lipid hydroperoxides level at the expense of GSH [15, 23–25]. AR has a K_m for NADPH 10-fold lower than glutathione reductase. AR could steal NADPH from glutathione reductase and cause decrease in the GSH level [24]. Other investigators reported that oxidation of sorbitol to fructose by SDH causes oxidative stress because its co-factor NAD⁺ is converted to NADH in the process, and NADH is the substrate for NAD(P)H oxidase to generate ROS [26]. Furthermore, the polyol pathway activity contributes to nonenzymatic glycation/glycoxidation by providing important glycation agents; fructose, fructose 3-phosphate, methylglyoxal, and 3-deoxyglucosone [17, 27, 28]. Because these are more potent non-enzymatic glycation agents than glucose, the flux of sorbitol pathway would result in increased AGE formation [29, 30]. A number of investigators suggested that hyperglycemia-induced GSH depletion occurs due to glycation or reduced expression of key enzymes of glutathione biosynthesis, γ -glutamyl cysteine synthetase and glycation of glutathione reductase [31, 32]. Thus, it is reasonable that

inhibition of the polyol pathway reduced oxidative stress.

Whereas lipid hydroperoxides levels in erythrocytes of diabetic patients were significantly reduced by epalrestat, this ARI did not alter other oxidative stress markers and antioxidants, TBARS, MDA-LDL, vitamin E, β -carotene in this study. These data support that the lipid hydroperoxides might be a clinical useful oxidative marker to evaluate various anti-atherosclerotic drugs. Different from other markers, we measured lipid peroxidation in erythrocyte membrane. Erythrocytes contain high concentrations of GSH and AR [33–35]. Thus, it is possible that the effect of ARI on oxidative stress is more easily detected in erythrocytes than serum. Lipid hydroperoxides are the primary products of lipid peroxidation and their detection has been frequently used as an effective tool for monitoring lipid peroxidation both *in vivo* and *in vitro*. Furthermore, the decrease in total lipid hydroperoxides suggests that a redox balance tends to reduce oxidative stress. On the other hand, the total volume of lipid hydroperoxides *per se* is merely partial aspect of the whole redox mechanisms. Further studies are needed to elucidate the clinical importance of measurement of lipid hydroperoxidases in erythrocytes.

A number of ARI have been developed, most of them have successfully shown positive effects in animal experiments, whereas the overall results of clinical

trials have yielded uncertain results, in part due to the unacceptable adverse effects or weak efficacy [36]. However, two recent studies on diabetic neuropathy demonstrated significant improvements in some of the disease symptoms in the drug-treated group [37, 38]. Considering the importance of oxidative stress in the pathophysiology of diabetic state and related complications, reduction of oxidative stress by ARI may mediate the observed effects.

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