

Research article

# The effect of ubiquinone and combined antioxidant therapy on oxidative stress markers in non-proliferative diabetic retinopathy: A phase IIa, randomized, double-blind, and placebo-controlled study

**Adolfo Daniel Rodríguez-Carrizalez<sup>1</sup>, José Alberto Castellanos-González<sup>2</sup>, Esaú César Martínez-Romero<sup>2</sup>, Guillermo Miller-Arevillaga<sup>2</sup>, Fermín Paul Pacheco-Moisés<sup>3</sup>, Luis Miguel Román-Pintos<sup>1</sup>, Alejandra Guillermina Miranda-Díaz<sup>1</sup>**

<sup>1</sup>Department of Physiology, Clinical and Experimental Treatment Institute, University Health Sciences Centre, University of Guadalajara, Mexico (Instituto de Terapéutica Experimental y Clínica, Departamento de Fisiología), <sup>2</sup>Department of Ophthalmology, Specialties Hospital of the National Occidental Medical Centre, Mexican Social Security Institute, Mexico (Hospital de Especialidades del Centro Médico Nacional de Occidente, Instituto Mexicano del Seguro Social), <sup>3</sup>University Centre of Exact and Engineering Sciences, University of Guadalajara, Mexico (Centro Universitario de Ciencias Exactas e Ingeniería)

**Objective:** To evaluate the effect of ubiquinone (Coenzyme Q10) and combined antioxidant therapy (CAT) on oxidative stress markers in non-proliferative diabetic retinopathy (NPDR) under clinical management.

**Study design:** In a randomized, double-blind, phase IIa, placebo-controlled, clinical trial, three study groups were formed and administered medications as follows: Group 1, Coenzyme Q10; Group 2, CAT; and Group 3, placebo.

**Methods:** Serum levels of the products of lipid peroxidation (LPO) and nitrites/nitrates, as markers of oxidative/nitrosative stress, were measured. As antioxidants, the total antioxidant capacity (TAC), catalase activity, and glutathione peroxidase (GPx) activity were measured.

**Results:** Baseline serum levels of LPO and nitrites/nitrates were significantly elevated in the three groups vs. healthy group ( $P < 0.0001$ ), while final levels in the Coenzyme Q10 and CAT groups were decreased vs. normal levels ( $P < 0.0001$ ). The baseline TAC was consumed in the three groups ( $P < 0.0001$ ), while final results in the Coenzyme Q10 and CAT groups improved ( $P < 0.0001$ ). Baseline catalase activity was increased in all groups vs. normal values ( $P < 0.001$ ), while final levels in the Coenzyme Q10 ( $P < 0.001$ ) and CAT groups ( $P < 0.0001$ ) were decreased. GPx behaved similarly to catalase and improved in the final results ( $P < 0.0001$ ).

**Discussion:** Adjunctive antioxidant treatment for 6 months was effective and safe for improving the oxidative stress in NPDR.

**Key words:** Antioxidants, Diabetes mellitus, Diabetic retinopathy, Nitrosative stress, Oxidative stress

## Introduction

Diabetic retinopathy (DR) is a progressive microangiopathy that produces lesions and occlusion of the retinal vessels. Non-proliferative diabetic retinopathy

(NPDR) is the state preceding proliferative diabetic retinopathy (PDR). PDR can cause detachment of the retina and provoke partial or total blindness. Sustained hyperglycemia is the highest risk factor related to development of this complication.<sup>1</sup> In prolonged exposure to high concentrations of glucose, retinal cells exhibit biochemical alterations in nutrient transfer to neuronal Müller cells.<sup>2</sup> Pericytes and retinal

Correspondence to: Alejandra Guillermina Miranda-Díaz, Department of Physiology, Clinical and Experimental Treatment Institute, University Health Sciences Centre, University of Guadalajara, Av. La Paz No. 2758, Col. Arcos Sur 44150, Guadalajara, Jalisco, México.  
Email: kindalex1@outlook.com

vascular apoptosis contribute to the development of acellular capillaries and phantom pericytes.<sup>3</sup> Hyperglycemia activates the following pathways that participate in DR formation: (a) polyol pathways; (b) activation of the protein K kinase; (c) accumulation of products of advanced glycation; (d) activation of hexosamine biosynthesis, growth factors, and oxidative stress.<sup>4</sup> Oxidative stress is the imbalance between pro-oxidants and antioxidants, and it affects carbohydrates, lipids, proteins, and nucleic acids.<sup>5</sup> The oxidative stress plays a pivotal role in cellular injury from hyperglycemia. High glucose level can stimulate reactive oxygen species (ROS) and reactive nitrogen species production. The defense system of the body becomes unable to counteract the enhanced ROS generation and, as a result, there is imbalance between ROS and their protective mechanisms that leads to domination of the condition of oxidative stress.<sup>6</sup>

In this study, we have considered malondialdehyde (MDA) and 4-hydroxy-alkenals (HNE) as markers of lipid peroxidation (LPO). LPO is a major harmful consequence of ROS formation, as it reflects irreversible oxidative changes of membranes. Increased serum concentrations of MDA and HNE have been found in patients with NPDR.<sup>7</sup> The hyperglycemia induces free radicals and impairs the endogenous antioxidant defense system in patients with diabetes. Endogenous antioxidant defense mechanisms include both enzymatic and non-enzymatic pathways. In this study, we measured the activities in serum of the enzymatic antioxidants catalase and glutathione peroxidase (GPx) and concurrently determined the values of LPO and NO metabolites. We assumed oxidative and nitrosative stress because of the imbalance between low serum total antioxidant capacity (TAC) and high quantities of MDA-HNE and nitrites/nitrates.

Under physiological conditions ROS are produced in low concentrations, but in chronic hyperglycemia their production is increased. The enzymatic antioxidant system of the human organism includes catalase, GPx, and superoxide dismutase (SOD). Vitamins C and E are involved in neutralizing ROS.<sup>8</sup> In the presence of superoxide anion, NO is transformed into peroxynitrite, causing nitrosative stress with the capacity to induce apoptosis of the blood-retinal barrier, to produce retinal damage.<sup>9</sup>

NPDR is not medically treated by specific means but merely by recommendations on improving glycaemic control.<sup>10</sup> To date, no medication is capable of stabilizing oxidative stress or preventing progression of microvascular damage in PDR.<sup>11</sup> Ubiquinone (Coenzyme Q10) is similar to quinone and is found in high concentrations in tissues with high-energy exchange (heart, brain, liver, and kidney).<sup>12</sup> In

Parkinson and Huntington diseases, a dose of 600–1,200 mg/day is recommended.<sup>13</sup> Coenzyme Q10 is reduced to ubiquinol in the intestine and is transported by a lymphatic route to the circulatory system. The absorption is poor owing to having a relatively large-molecular weight, and it being insoluble in water and sparingly, in its crystalline form.<sup>14,15</sup> It is distributed among lipoproteins to protect them from oxidation. The sub-cellular distribution (40–50%) is in the internal mitochondrial membrane, and it has a half-life of 33 hours and an area below the curve of 44.94–64.01  $\mu\text{mol h/l}$ .<sup>16</sup> The administration of combined antioxidant therapy (CAT) (Drusen Laz in Mexico, SIFI Laboratories) contributes by improving antioxidant capacity against ROS and protecting photo-receptors against radiation. Vitamins C and E act by normalizing numerous chemical reactions to diminish aging and degeneration caused by ROS. CAT is recommended in macular degeneration.<sup>17</sup> No information was found on the effects of CAT in NPDR. Adverse effects of Coenzyme Q10 and CAT are infrequent.

The objective of this study was to evaluate the effects of Coenzyme Q10 and CAT on oxidative stress and antioxidant markers in NPDR during clinical management.

## Material and methods

A randomized, double-blind, phase IIa placebo-controlled, clinical trial was carried out. From patients with type 2 diabetes mellitus (DM), 60 with NPDR, but without macular edema, were selected, and randomized in three groups ( $n = 20$  patients per group). Patients and investigators were unaware as to which group each patient was assigned until the end of the study.

Three interventional adjuvant treatment groups were formed: Group 1, treated with 400 mg of Coenzyme Q10;<sup>18</sup> Group 2, treated with a CAT tablet (composed of 10 mg of lutein, 4 mg of astaxanthin, 1 mg of zeaxanthin, 180 mg of vitamin C, 30 mg of vitamin E, 20 mg of zinc, and 1 mg of copper);<sup>19</sup> and Group 3, treated with a placebo. The pharmacological administration was a single daily dose for 6 months. Twenty healthy subjects of similar age and gender were included as a control group to standardize the normal values of the study parameters (blood donors from the blood bank who agreed to donate 10 ml extra blood in addition to the amount donated). Compliance was measured by an independent investigator (a pharmacist), external to the study, who had the responsibility of measuring treatment adherence with a medication log; in each clinical visit this person did the counting of the medications, to quantify the drug ingested from each group, every month. He considered the minimum compliance

as 80% ingestion of the medications, and dispensed the medications for the following month, throughout the study. Another member of the research team, a clinical monitor, evaluated the correct form of the data register in the case report format.

Patients were followed by their family physician who managed their DM and diets (a diabetic diet and physical activity was recommended to them according to the individual characteristics of each patient). In this study, the patients underwent pharmacological intervention with antioxidants, like an adjuvant therapy, in combination with hypoglycemic, antihypertensive, and hypolipidemic drugs. In addition, the family physician clinic followed the medical nutrition therapy recommendations for the management of adults with DM and the physical activity considerations with diabetes complications, both according to the American Diabetes Association (ADA) guidelines.<sup>20</sup>

Adherence to treatment of DM and the nutritional and physical activity recommendations depended on each patient. Included were males and females, with glycated hemoglobin (HbA1c)  $\leq 12\%$ . Vigilance for appearance of adverse reactions followed the Official Mexican Guidelines (*Norma Oficial Mexicana: NOM-220-SSA1-2012*). Fasting glucose, HbA1c to monitor metabolic control, and hepatic and renal enzymes, lipid profiles, and general urine exams were evaluated in order to determine the safety of the medication used.

Blood pressure was measured according to the recommendations of the American Heart Association using a mercury sphygmomanometer. After 15 minutes of rest, three consecutive measurements were taken at 2-minute intervals. The average of the first, the second, and third measurement was then calculated and registered.<sup>21</sup>

Ocular parameters were assessed at baseline and at the 6-month follow-up by recording the best-corrected visual acuity (CVA), intraocular pressure, slit-lamp examination, retinal examination, stereo color fundus photographs, and fundus fluorescein angiography (FFA).<sup>22</sup> Best-CVA was recorded by Snellen's visual acuity chart and analysis performed using an equivalence system expressed in decimals from 0.1 to 1.0.<sup>23</sup> Intraocular pressure was recorded with the Goldmann applanation tonometer by an ophthalmologist. Slit-lamp examination details including the presence or absence of cataract were documented. Pupils of the subjects were dilated with tropicamide 1%. After dilation of the pupils, detailed fundus examination was performed by an ophthalmologist using direct and indirect ophthalmoscopy. Fundus examination included a slit-lamp biomicroscopy with 90 D or 78 D lenses. Seven-field color retinal photography was performed with the Zeiss FF 450 camera (Carl Zeiss Ltd, Jena, Germany) using 35 mm color

transparencies. The areas photographed included stereo pictures of the macula, disk, temporal to the macula, superior-temporal, inferior-temporal, superior-nasal, and inferior-nasal areas. The photographs were assessed and graded using the international DR and diabetic macular edema disease severity scales. FFA was performed in all cases before (baseline) and after (final) antioxidant therapy. The need for additional treatment was decided by the clinical presentation, which was documented by visual acuity, color photographs, and FFA at baseline and at 6-month follow-up examinations by the ophthalmologist according to the guidelines provided in the Early Treatment Diabetic Retinopathy Study.<sup>24</sup>

#### *Blood samples*

Peripheral blood samples (10 ml) were obtained from all study participants after an 8-hour overnight fast. Sera were separated and stored at  $-80^{\circ}\text{C}$  until processing.

#### *Products of lipid peroxidation*

Levels were measured according to the manufacturer's specifications (Oxford Biomedical Research, Inc., FR12<sup>®</sup>). The chromogen reagent reacts with MDA and 4-HDE to form a stable chromophore: 200  $\mu\text{l}$  of serum with 455  $\mu\text{l}$  of *N*-methyl-2-phenylindole in acetonitrile (Reagent 1) was diluted with ferric acid in methanol. Samples were agitated, and 105  $\mu\text{l}$  of methanesulfonic acid was added; incubated at  $45^{\circ}\text{C}$  for 60 minutes, and centrifuged at 12,791 rpm for 10 minutes. Two-hundred microlitres of the supernatant was taken and absorbance was measured at 586 nm. A standard curve with known concentrations of 1,1,3,3-tetramethoxypropane in Tris-HCl was established.

#### *NO metabolites*

Prior to the measurement of the NO levels, the samples were deproteinized through the addition of zinc sulfate.<sup>25</sup> The colorimetric method was used as per the kit's instructions (Nitric Oxide Assay Kit, User Protocol 482650, Calbiochem<sup>®</sup>). Eight-five microlitres of standard or sample was placed in wells and 10  $\mu\text{l}$  of nitrate reductase and 10  $\mu\text{l}$  of 2 mM NADH were added. The plate was agitated for 20 minutes at room temperature, 50  $\mu\text{l}$  of colorant 1 was added and agitated, and 50  $\mu\text{l}$  of colorant 2 was added, agitated at room temperature, and read at 540 nm.

#### *Total antioxidant capacity*

TAC was measured according to the kit's instructions (Total Antioxidant Power Kit, No. TA02.090130, Oxford Biomedical Research<sup>®</sup>), to obtain a concentration in mM equivalents of uric acid. Standards

and samples were diluted 1:40, and 200- $\mu$ l aliquots were placed in each well. The plate was read at 450 nm as a reference value, and then 50  $\mu$ l of copper solution was added and incubated for 3 minutes at room temperature; 50  $\mu$ l of stop solution was added and read at 450 nm.

### Catalase

Catalase was determined by kit according to the manufacturer's instructions (Bioxytech Catalase-520<sup>®</sup>, cat. 21042, OXIS Int, Beverly Hills, CA, USA), where 30  $\mu$ l of diluted standard or sample and 500  $\mu$ l of substrate (10 mM of H<sub>2</sub>O<sub>2</sub>) were added, then erythrocytes were incubated for 1 minute at room temperature, and 500  $\mu$ l of stop reagent was added. The sample/standard was covered, mixed, and immersed, and 20  $\mu$ l of each reagent was added to the tubes. Two milliliters of HRP/reactive chromogen was deposited, mixed in a water bath, and incubated for 10 minutes at room temperature. The absorbance was read at 520 nm.

### Glutathione peroxidase

Measurement of the GPx activity was done according to the manufacturer's instructions (Bioxytech GPx-340<sup>®</sup>, cat. 21017). The reagent was based on the oxidation of reduced glutathione in the presence of *tert*-butyl hydroperoxide, glutathione reductase, and NADPH. The decrease in absorbance at 340 nm following substrate addition was recorded; the rate of decrease in absorbance is directly proportional to GPx activity.

### Statistical analysis

Results are expressed as mean  $\pm$  standard error. Qualitative variables were determined with frequencies and percentages, and the Chi<sup>2</sup> test. The power calculation was 90%, the confidence interval was 95%.  $Z\alpha = 1.969$ , alpha value = 0.05,  $Z\beta = 1.282$ , and beta value = 0.10. All the data were computed with Excel and statistical analyses were done using SPSS PC windows version 21 (Chicago, IL, USA). The Mann-Whitney test was used to compare continuous variables between groups and the Wilcoxon test was used to compare continuous variables within the group. *P*-values of <0.05 were considered significant. Multivariate logistic regression analysis was performed to determine the risk factors associated with decreased LPO and NO from baseline at the 6-month follow-up. Decreased LPO and NO were used as the dependent variable, and the independent variables included age, body mass index, duration of diabetes, systolic and diastolic BP, HbA1c, total cholesterol, serum triglyceride, HDL-cholesterol, and LDL-cholesterol at baseline. To determine the risk factors of NPDR, stepwise multivariate logistic regression analysis also was performed using NPDR as the dependent variable and the risk factors, age,

gender, body mass index, duration of diabetes, fasting blood glucose, HbA1c, total cholesterol, serum triglyceride, HDL-cholesterol, and LDL-cholesterol at baseline as independent variables.

### Ethical considerations

The study was approved by the National Research and Ethics Committee of the Mexican Social Security Institute (R-2012-785-040); the Research Ethics and Bioethics Committee of the University Health Sciences Centre at the University of Guadalajara (Folio C.I.2010); the State Health Research Registry (62/UG-JAL/2011); to eliminate ClinicalTrials.gov (Identifier: NCT02062034), in agreement with guidelines as stipulated by the Declaration of Helsinki, the Good Clinical Practices Guide, and the International Conference on Harmonization for Research in Human Beings (64th General Assembly, Fortaleza, Brazil, October 2013). Patients signed their informed consent and were informed of the results. Their confidentiality was maintained.

### Results

Baseline values in the three groups were not different when comparing averages of the clinical parameters, which indicated that groups were similar, comparable, and without selection bias. Patients had suffered from DM for >14 years, and were overweight (Table 1).

### Metabolic profiles

The three groups had persistent hyperglycemia throughout the study period despite metabolic control as guided by their family physician, nutritional recommendations, and insistence on a healthy lifestyle. They did not achieve the therapeutic goals in control of their glycemia. At the end of the study, there was a significant decrease in HbA1c in the Placebo group (from  $9.22 \pm 0.39$  to  $8.01 \pm 0.4\%$ ,  $P < 0.048$ ) and in the CAT group (from  $9.6 \pm 0.4$  to  $8.6 \pm 0.2\%$ ,  $P < 0.05$ ), which suggests a favorable outcome in lifestyle modifications. In the Coenzyme Q10 group, there were no changes to HbA1c. Lipid profiles were unaltered between baseline and final results (Table 2).

### Products of lipid peroxidation

Normal levels of MDA + 4-HDE were  $0.98 \pm 0.13 \mu\text{mol/l}$ . Baseline levels in the three study groups were significantly elevated vs. normal levels ( $P < 0.0001$ ): Placebo group,  $2.69 \pm 0.16 \mu\text{mol/l}$ ; Coenzyme Q10 group,  $2.39 \pm 0.13 \mu\text{mol/l}$ ; and CAT group  $2.75 \pm 0.16 \mu\text{mol/l}$ . At the end of the study, LPO levels decreased significantly in groups exposed to antioxidant therapy ( $P < 0.0001$ ): Coenzyme Q10 group,  $1.54 \pm 0.10 \mu\text{mol/l}$ ; and CAT group,  $1.16 \pm 0.12 \mu\text{mol/l}$ . Results were unchanged in the Placebo group,  $2.79 \pm 0.15 \mu\text{mol/l}$  (Table 3).

**Table 1 Clinical characteristics of the study groups**

		Placebo (n = 20)			Coenzyme Q10 (n = 20)			Combined antioxidant therapy (n = 20)			
		Baseline	Final	P WCX	Baseline	Final	P WCX	Baseline	Final	P WCX	P K-W
Gender	%	(9/11)		–	(11/9)		–	(10/11)		–	–
(male/ female)											
Age	Years	57.8 ± 1.9		–	58.5 ± 1.9		–	62.1 ± 1.1		–	NS
DM type 2 evolution	Years	14.2 ± 1.3	14.2 ± 1.3	–	15.4 ± 1.6	15.4 ± 1.6	–	15.2 ± 1.3	15.2 ± 1.3	–	NS
Body mass index (BMI)	kg/m <sup>2</sup>	29.3 ± 0.8	29.2 ± 0.9	NS	28.2 ± 3.7	28.5 ± 0.9	NS	28.6 ± 4.5	29.2 ± 0.9	NS	NS
Systolic arterial pressure (SAP)	mmHg	128.4 ± 1.3	129.5 ± 2.7	NS	131.4 ± 19.4	132.4 ± 3.2	NS	144.5 ± 15.8	135.8 ± 3.5	NS	NS
Diastolic arterial pressure (DAP)	mmHg	74.0 ± 1.1	75.1 ± 2.0	NS	80.7 ± 10.8	73.4 ± 2.7	NS	84.8 ± 7.2	74.9 ± 2.8	NS	NS
Left eye pressure (LEP)	mmHg	14.9 ± 2.1	14.8 ± 0.6	NS	15.4 ± 1.1	15.2 ± 0.5	NS	15.3 ± 0.9	14.7 ± 0.9	NS	NS
Corrected visual acuity (CVA)	6 m	0.9 ± 0.0	0.9 ± 0.0	NS	0.8 ± 0.0	0.8 ± 0.0	NS	0.8 ± 0.1	0.8 ± 0.1	NS	NS

The table illustrates the metabolic profile per study group, where values are expressed as mean ± standard error. The three groups were homogeneous. The *P*-value was calculated with the Wilcoxon and Kruskal–Wallis tests, with no significant differences.

### Nitrites/nitrates

Normal levels were 12.31 ± 1.15 pmol/ml. Baseline levels in the groups were significantly increased compared to normal values (*P* < 0.0001): Placebo group, 38.77 ± 1.89 pmol/ml; Coenzyme Q10 group, 34.54 ± 1.59 pmol/ml; and CAT group, 39.68 ± 2.06 pmol/ml. At the end, levels had decreased significantly (*P* < 0.0001): in the Coenzyme Q10 group, 26.24 ± 2.25 pmol/ml, and CAT group, 28.06 ±

1.60 pmol/ml. Levels in the Placebo group remained similar to baseline, at 38.90 ± 1.51 pmol/ml (Table 3).

### Total antioxidant capacity

The TAC offers a complete panorama of the function of antioxidant systems in the organism. Normal levels were 22.41 ± 1.10 mMeq of uric acid/ml. Baseline results were significantly diminished in the three study groups compared to normal values (*P* <

**Table 2 Metabolic profile in NPDR**

	Placebo (n = 20)			Coenzyme Q10 (n = 20)			Combined antioxidant therapy (n = 20)			
	Baseline	Final	P WCX	Baseline	Final	P WCX	Baseline	Final	P WCX	P K-W
Glycated hemoglobin (HbA1c, %)	9.22 ± 0.39	8.01 ± 0.4	0.048	8.5 ± 0.4	8.3 ± 0.4	NS	9.6 ± 0.4	8.6 ± 0.2	0.05	NS
Glucose (mg/dl)	125.2 ± 8.0	135.3 ± 12.4	NS	149.1 ± 12.9	135.7 ± 11.0	NS	149.4 ± 13.5	124.7 ± 10.2	NS	NS
Total cholesterol (TC, mg/dl)	200.6 ± 7.1	195.5 ± 7.1	NS	176.8 ± 7.4	169.2 ± 9.8	NS	205.0 ± 8.1	200.4 ± 8.6	NS	NS
Low-density cholesterol (LDL, mg/dl)	129.5 ± 15.6	115.9 ± 7.8	NS	90.3 ± 5.2	94.3 ± 5.7	NS	114.8 ± 7.1	110.6 ± 6.9	NS	NS
High-density cholesterol (HDL, mg/dl)	47.3 ± 3.5	46.5 ± 2.3	NS	42.9 ± 2.1	46.1 ± 2.6	NS	41.8 ± 1.1	46.9 ± 2.6	NS	NS
Triglycerides (TGC, mg/dl)	214.3 ± 22.4	180.4 ± 17.6	NS	210.0 ± 28.5	198.5 ± 20.4	NS	252.8 ± 27.3	223.4 ± 25.7	NS	NS

The table illustrates metabolic profile per study group, where values are expressed as mean ± standard error. There was a significant decrease in HbA1c in the Placebo (*P* < 0.048) and combined antioxidant therapy groups (*P* < 0.05). These improvements could be due to close medical attention or lifestyle changes.

NPDR, non-proliferative diabetic retinopathy; HbA1c, glycated hemoglobin, %, percent, TC, total cholesterol; LDC, low-density cholesterol; HDL, high-density cholesterol; TGC, triglycerides; mg/dl, milligram/deciliter, NS, not significant; WCX, Wilcoxon test, K–W, Kruskal–Wallis test.

Table 3 Oxidants and antioxidants

	Normal value	Placebo (n = 20)			Coenzyme Q10 (n = 20)			Combined antioxidant therapy (n = 20)		
		Baseline	Final	P WCX	Baseline	Final	P WCX	Baseline	Final	P WCX
LPO (MDA-4-HDE, $\mu\text{M}/\text{l}$ )	0.98 $\pm$ 0.13	2.69 $\pm$ 0.16	2.79 $\pm$ 0.15	NS	2.39 $\pm$ 0.13	1.54 $\pm$ 0.10	0.0001	2.75 $\pm$ 0.16	1.16 $\pm$ 0.12	0.0001
Nitrites/Nitrites (pmol/ml)	12.31 $\pm$ 1.15	38.77 $\pm$ 1.89	38.90 $\pm$ 1.51	NS	34.54 $\pm$ 1.59	26.24 $\pm$ 2.25	0.005	39.68 $\pm$ 2.06	28.06 $\pm$ 1.60	0.0001
Total antioxidant capacity (TAC, nM)	22.41 $\pm$ 1.10	11.90 $\pm$ 0.58	9.81 $\pm$ 0.45	0.0001	12.51 $\pm$ 0.70	15.50 $\pm$ 0.63	0.0001	11.30 $\pm$ 0.72	14.61 $\pm$ 0.60	0.0001
Catalase (U/mg)	94.17 $\pm$ 1.58	131.93 $\pm$ 3.54	136.86 $\pm$ 6.69	NS	129.95 $\pm$ 3.90	105.63 $\pm$ 5.10	0.0001	138.18 $\pm$ 4.61	106.72 $\pm$ 4.37	0.0001
Glutathione peroxidase (GPx, U/min/mg)	35.13 $\pm$ 2.74	113.44 $\pm$ 14.02	119.71 $\pm$ 7.30	NS	116.49 $\pm$ 12.73	45.20 $\pm$ 7.61	0.0001	99.89 $\pm$ 8.09	43.14 $\pm$ 5.16	0.0001

The table illustrates the significant decrease in LPO, nitrites/nitrites, and TAC in patients managed for 6 months with Coenzyme Q10 and combined antioxidant therapy. The activity of the antioxidant enzymes catalase and GPx tended to significantly regularize toward normal limits.

LPO, lipoperoxidation products; MDA, malondialdehyde; 4-HDA, 4-hydroxy-alkenals; P, statistical value; pmol/ml, picomol/milliliter; nM, nanomolar; TAC, total antioxidant capacity; U/mg, Units/milligram; GPx, glutathione peroxidase; U/min/mg, units/minute/milligram; WCX, Wilcoxon test; K-W, Kruskal-Wallis test; NS, not significant.

0.0001): Placebo, 11.90  $\pm$  0.58 mM; Coenzyme Q10, 12.51  $\pm$  0.70 mM; and CAT, 11.30  $\pm$  0.72 mM. At the end, TAC increased significantly in groups subjected to pharmacological antioxidant therapy ( $P < 0.0001$ ): Coenzyme Q10, 15.50  $\pm$  0.63 mM and CAT, 14.61  $\pm$  0.60 mM. In the Placebo group, levels remained low, 9.81  $\pm$  0.45 mM (Table 3).

### Catalase activity

Normal activity of the enzyme was 94.17  $\pm$  1.58 U/mg of protein. Baseline activity was significantly increased in the groups vs. the normal activity, possibly in compensation for the decrease in TAC: Placebo group, 131.93  $\pm$  3.54 U/mg; Coenzyme Q10 group, 129.95  $\pm$  3.90 U/mg; and CAT group, 138.18  $\pm$  4.61 U/mg ( $P < 0.001$ ). At the end, the activity decreased significantly ( $P < 0.002$ ) in groups exposed to antioxidant therapy: Coenzyme Q10, 105.63  $\pm$  5.10 U/mg ( $P < 0.001$ ) and CAT, 106.72  $\pm$  4.37 U/mg ( $P < 0.0001$ ). Levels in the Placebo group remained elevated at 136.86  $\pm$  6.69 U/mg, perhaps owing to the constant presence of nitrosative and oxidative stress (Table 3).

### Glutathione peroxidase activity

Normal activity was 35.13  $\pm$  2.74 U/min/mg of protein. Initial GPx enzyme activity found in the groups was significantly increased: the Placebo group, 113.44  $\pm$  14.02 U/min/mg ( $P < 0.001$ ); Coenzyme Q10 group, 116.49  $\pm$  12.73 U/min/mg ( $P < 0.001$ ); and the CAT group, 99.89  $\pm$  8.09 U/min/mg ( $P < 0.002$ ). At the end of the study, the activity of the enzyme was decreased significantly with a tendency to normalize in groups subjected to antioxidant therapy ( $P < 0.0001$ ): Coenzyme Q10 group, 45.20  $\pm$  7.61 U/min/mg and the CAT group, 43.14  $\pm$  5.16 U/min/mg. The Placebo group sustained increased activity with 119.71  $\pm$  7.30 U/min/mg. Derregulation of the antioxidant enzymes could have been due to the presence of oxidative and nitrosative stress in the persistent hyperglycemic state (Table 3).

There was a positive correlation (Spearman's correlation test) between high levels of LPO and NO ( $P < 0.0001$ ), as well as a negative correlation between these markers and consumption of TAC ( $P < 0.0001$ ), and between high levels of NO and consumption of TAC ( $P < 0.001$ ).

### Safety profile

Safety of the medications was considered extremely important when comparing baseline vs. final values, without significant alterations being detected by the Wilcoxon test, which supports the likelihood that medications were safe, with no adverse effects reported (Table 4).

**Table 4 Safety profile of patients with NPDR**

	Placebo (n = 20)			Coenzyme Q10 (n = 20)			Combined antioxidant therapy (n = 20)			
	Baseline	Final	P WCX	Baseline	Final	P WCX	Baseline	Final	P WCX	P K-W
Uric acid (mg/dl)	5.6 ± 0.3	4.9 ± 0.3	NS	5.1 ± 0.2	4.9 ± 0.2	NS	5.7 ± 0.2	5.6 ± 0.3	NS	NS
TB (mg/dl)	0.6 ± 0.1	0.6 ± 0.2	NS	0.5 ± 0.0	0.6 ± 0.1	NS	0.5 ± 0.0	0.5 ± 0.0	NS	NS
DB (mg/dl)	0.2 ± 0.0	0.3 ± 0.0	NS	0.3 ± 0.0	0.2 ± 0.0	NS	0.2 ± 0.0	0.2 ± 0.0	NS	NS
IB (mg/dl)	0.4 ± 0.0	0.3 ± 0.0	NS	0.3 ± 0.0	0.4 ± 0.1	NS	0.3 ± 0.0	0.4 ± 0.0	NS	NS
Urea (mg/dl)	36.1 ± 3.5	35.9 ± 4.1	NS	32.6 ± 2.6	32.5 ± 1.6	NS	37.2 ± 2.8	41.9 ± 4.2	NS	NS
Cr (mg/dl)	0.9 ± 0.1	0.9 ± 0.1	NS	0.8 ± 0.1	0.8 ± 0.0	NS	0.9 ± 0.1	1.0 ± 0.1	NS	NS
AST (IU/l)	28.0 ± 2.3	24.0 ± 1.5	NS	29.8 ± 2.5	29.4 ± 2.9	NS	28.9 ± 1.5	24.6 ± 1.4	NS	NS
ALT (IU/l)	31.2 ± 1.9	30.2 ± 1.9	NS	34.6 ± 3.1	36.7 ± 3.7	NS	30.3 ± 1.9	30.1 ± 1.6	NS	NS

The table illustrates the metabolic profile per study group, where values are expressed as mean ± standard error. Significant differences were not observed between study groups, which suggests that the medications evaluated were safe (Wilcoxon and Kruskal–Wallis tests).

NPDR, non-proliferative diabetic retinopathy; mg/dl, milligram/deciliter; IU/l, international units per liter; WCX, Wilcoxon test; K–W, Kruskal–Wallis test; NS, not significant.

## Discussion

Despite the fact that patients included in this study were systematically managed by their family physician in a primary intervention plan that aimed to correct their lack of metabolic control, it was not possible to lower glucose levels; this perhaps was due to the chronicity of their DM, dietary transgressions, or through lack of adherence to concomitant medications for treatment of metabolic diseases. Nutritional plan and physical activity regimens were recommended to patients based on their individual characteristics according ADA Guidelines. The study was performed on ambulatory patients. Compliance about non-pharmacological recommendations and adherence to treatment of diabetes were evaluated by the family doctor, not by the researchers.<sup>26</sup>

The presence of DR is strongly related to the duration of diabetes, with an approximate three-fold increase in vision-threatening DR in those who have had DM for 10 years or more.<sup>27</sup> There was a significant decrease in HbA1c in the Placebo and CAT groups, which suggests a beneficial outcome through lifestyle modifications, self-care, and close vigilance by the treating physician. In the Coenzyme Q10 group, the HbA1c remained unchanged, contrary to a report by Brauner *et al.*<sup>28</sup> However, other investigators did not find a decrease in hyperglycemia and HbA1c.<sup>29</sup> Dysglycemia involves two components that participate in augmenting oxidative stress: (a) sustained, chronic hyperglycemia through excessive glycosylation of proteins on activating oxidative stress; and (b) acute glucose fluctuations that can have effects more toxic than sustained hyperglycemia in the development of diabetic complications, with the ability to also unchain oxidative stress.<sup>30</sup>

We found a significant increase in baseline serum levels of LPO in the three groups of patients vs. normal values. Other authors also have reported a notable increase in LPO in PDR animal experiments.<sup>9</sup>

Recently, a study reported a relationship between progressive increases in LPO and severity of NPDR<sup>31</sup>, which can cause progression to PDR. LPO are sensitive and trustworthy markers, widely accepted as determinants of cellular membrane damage induced by oxidative stress. Diverse LPO markers are available with different grades of specificity. MDA is a global marker, F(2) isoprostane forms specifically from arachidonic acid, and the 4-HDEs are derived from hydroperoxides of the *n*-3 fatty acids. All effectively offer a panorama of the LPO state.<sup>32</sup> The aldehydes most intensively studied are 4-HDE and MDA.<sup>33</sup> A recent study reported that the isoprostanes attenuate the liberation of dopamine *in vitro* in the neural retina of mammals.<sup>34</sup> One weakness of this study is in not having measured the isoprostanes.

It must be emphasized that retinal cells are highly sensitive to oxidative damage caused by the constant photochemical reactions, and the high concentrations of polyunsaturated fatty acids that constitute their membranes are directly affected by LPO. Ingestion of Coenzyme Q10 and CAT was significantly superior for decreasing the LPO levels, to values closer to normal, an outcome similar to that reported recently in *vitreous* humor.<sup>35</sup>

Likewise, serum levels of nitrites/nitrates in NPDR were significantly augmented compared to normal levels. In previous studies, an increase in serum levels of NO in PDR was seen compared to subjects with NPDR, without retinopathy, and in healthy controls.<sup>36</sup> The severity of DR is associated with an increase in systemic NO.<sup>37</sup> The increase in NO is a situation that has special relevance since before an increase in nitrite/nitrate metabolites occurs, inflammatory phenomena are involved that stimulate the production of nitrosative stress.<sup>38</sup> Chronic hyperglycemia causes damage to retinal vessels and endothelial dysfunction by increasing the adhesion of leukocytes and overproduction of NO.<sup>39</sup> However, the 6-month

exposure to antioxidant treatment (Coenzyme Q10 or CAT) was able to significantly diminish NO levels and decrease NO bioavailability.

We found the TAC is consumed in NPDR, compared to values determined in healthy subjects. The decreased TAC indicates higher oxidation, probably due to LPO and an increase in NO. The serum TAC is a complex system that results from the endogenous, antioxidant enzyme activity that includes SOD, catalase, GPx, the antioxidant vitamins C, and E, beta carotenes, albumin, ferritin, ceruloplasmin, uric acid, and bilirubin. Recently, low levels of TAC in *vitreous* humor were reported in diabetic patients, and much lower levels in DR.<sup>40</sup> In our study, we found a baseline TAC consumption and significant improvements at the end, which indicates a beneficial effect of the administered medications as adjuncts in improving antioxidant systems in hyperglycemia.<sup>41,42</sup>

Furthermore, activities of catalase and GPx were measured in erythrocytes, where high activity was found prior to antioxidant treatment in the study groups, possibly in compensation for the imbalance between oxidants and antioxidants. Interestingly, catalase and GPx activities decreased significantly at the 6-month follow-up in groups managed with Coenzyme Q10 and CAT, with a tendency for the antioxidant status to normalize. We consider that both antioxidant alternatives were safe and effective for correcting antioxidant enzyme activity, as was previously reported by our group in a transverse study.<sup>7</sup> Coenzyme Q10 and CAT significantly suppress ROS generation, tending to correct the activity of the systemic antioxidants. We have found an important imbalance between oxidants and antioxidants in NPDR. The adjunctive treatment of NPDR with oral antioxidants (Coenzyme Q10 and CAT) for 6 months provided benefits by correcting the oxidative/nitrosative stress. We are motivated to further investigate the effect of diminishing growth factor secretion in the vascular endothelium in hyperglycemic conditions.

### Acknowledgements

The authors thank Rogelio Troyo Sanromán, MSc., Professor of Science, for his help in the statistical analysis.

### Disclaimer statements

**Contributors** None.

**Funding** CANIFARMA 2013; Annual Award.

**Conflicts of interest** The author(s) declare(s) that there are no conflict of interests regarding the publication of this paper. The authors claim sole responsibility for the concepts expressed in this paper.

**Ethics approval** The study was approved by: the National Research and Ethics Committee of the Mexican Social Security Institute (R-2012-785-040); the Research Ethics and Bioethics Committee of the University Health Sciences Centre at the University of Guadalajara (Folio C.I.2010); by the State Health Research Registry (62/UG-JAL/2011); and, in ClinicalTrials.gov (Identifier: NCT02062034), in agreement with guidelines as stipulated by the Declaration of Helsinki, the Good Clinical Practices Guide, and the International Conference on Harmonization for Research in Human Beings (64th General Assembly, Fortaleza, Brazil, October 2013). Patients signed their informed consent and were informed of the results. Their confidentiality was maintained.

### References

- Engerman R, Kern T. Hyperglucemia as a cause of retinopathy. *Metabolism* 1986;35:20–23.
- Zhou ZJ, McCall MA. Retinal ganglion cells in diabetes. *AM J Physiol* 2008;586:4343–45.
- Kern TS, Tang J, Mizutani M, Kowluru RA, Nagaraj RH, Tomeo G, *et al.* Response of capillary cell death to aminoguanidine predicts the development of retinopathy comparison of diabetes and galactosemia. *Invest Ophthalmol Vis Sci* 2000;41:3972–78.
- Lorenzi M. The polyol pathway as a mechanism for diabetic retinopathy: attractive elusive, and resilient. *Exp Diabetes Res* 2007;2007:1–10.
- El-Asrar AM. Role of inflammation in the pathogenesis of diabetic retinopathy. *Middle East Afr J Ophthalmol* 2012;19(1):70–74.
- Tiwari B, Pandey K, Abidi A, Rizvi S. Markers of oxidative stress during Diabetes mellitus. *J Biomarkers* 2013;1–8.
- Rodríguez A, Castellanos J, Martínez E, Miller G, Pacheco F, Miranda A, *et al.* Oxidants, antioxidants and mitochondrial function in non-proliferative diabetic retinopathy. *J Diab* 2014;6(2):167–75.
- Guichardant M, Chantegrel B, Deshayes C, Doutheau A, Moliere P, Lagarde M. Specific markers of lipid peroxidation issued from n-3 and n-6 fatty acids. *Biochem Soc Trans* 2004;32(1):139–40.
- Kowluru R, Pooi-See C. Oxidative stress and diabetic retinopathy. *Exp Diabetes Res* 2007;2007:1–12.
- Wong T, Gillies M, Mohamed Q. Management of diabetic retinopathy a systematic review. *JAMA* 2007;298(8):902–16.
- Claramunt J. Retinopatía diabética. *Rev Med Clin CONDES* 2009;20(5):670–9.
- Bonakdar R, Guarneri E. Coenzyme Q10. *Am Fam Physician* 2005;72(6):1065–70.
- Crane F. Biochemical functions of coenzyme Q10. *J Am Coll Nutr* 2001;20(6):591–8.
- Kiebertz K. A randomized clinical trial of coenzyme Q10 and GPI-1485 in early Parkinson disease. *Neurology* 2007;68:20–28.
- Potgleter M, Pretorius E, Pepper M. Primary and secondary coenzyme Q10 deficiency: the role of therapeutic supplementation. *Nutr Rev* 2013;7(3):180–8.
- Mancuso M, Orsucci D, Volpi L, Calsolaro V, Siciliano G. Coenzyme Q10 in neuromuscular and neurodegenerative disorders. *Curr Drug Targets* 2010;11(1):111–21.
- Parisi V, Tedeschi M, Gallinaro G, Varano M, Saviano S, Piermarocchi S, CARMIS Study Group. Carotenoids and antioxidants in age-related maculopathy Italian study: multifocal electroretinogram modifications after 1 year. *Ophthalmology* 2008;115(2):324–33.
- Hernández-Ojeda J, Cardona-Muñoz EG, Román-Pintos LM, Troyo-Sanromán R, Ortiz-Lazareno PC, Cárdenas-Meza MA, *et al.* The effect of ubiquinone in diabetic polyneuropathy: a randomized double-blind placebo-controlled study. *J Diab Complicat* 2012;26(4):352–8.
- Piermarocchi S, Saviano S, Parisi V, Tedeschi M, Panozzo G, Scarpa G, *et al.* Carmis Study Group. Carotenoids in age-related maculopathy Italian Study (CARMIS): two-year

- results of a randomized study. *Eur J Ophthalmol* 2012;22(2): 216–25.
- 20 Standards of medical care in diabetes 2012. *Diabetes Care* 2012; 35(1):S11–S63.
  - 21 Drozda J, Jr, Messer JV, Spertus J, Abramowitz B, Alexander K, Beam CT, *et al.* ACCF/AHA/AMA-PCPI 2011 performance measures for adults with coronary artery disease and hypertension: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Performance Measures and the American Medical Association-Physician Consortium for Performance Improvement. *Circulation* 2011;124(2):248–70.
  - 22 Wilkinson CP, Ferris FL, III, Klein RE, Lee PP, Agardh CD, Davis M, *et al.* Global Diabetic Retinopathy Project Group. Proposed international diabetic retinopathy and diabetic macular edema disease severity scales. *Ophthalmology* 2003; 110:1677–82.
  - 23 Lima V. Retinopatía diabética simplificada: la escala clínica internacional. *Rev Hosp Jua Mex* 2006;73(4):170–4.
  - 24 Early Treatment Diabetic Retinopathy Study Research Group. ETDRS report no. 3: Techniques for scatter and focal photocoagulation treatment of diabetic retinopathy. *Int Ophthalmol Clin* 1987;274:254–64.
  - 25 Asghar Ghasemi A, Hedayati M, Biabani H. Protein precipitation methods evaluated for determination of serum nitric oxide end products by the Griess assay. *JMSR* 2007;2: 29–32.
  - 26 Standards of Medical Care in Diabetes 2012. *Diabetes Care* 2012;35(1):S11–63.
  - 27 Park CY, Park SE, Bae JC, Kim WJ, Park SW, Ha MM, *et al.* Prevalence of and risk factors for diabetic retinopathy in Koreans with type II diabetes: baseline characteristics of Seoul Metropolitan City-Diabetes Prevention Program (SMC-DPP) participants. *Br J Ophthalmol* 2012;96:151–5.
  - 28 Brauner H, Lüthje P, Grünler J, Ekberg NR, Dallner G, Brismar K, *et al.* Markers of innate immune activity in patients with type 1 and type 2 diabetes mellitus and the effect of the anti-oxidant coenzyme Q10 on inflammatory activity. *Clin Exp Immunol* 2014;177(2):478–82.
  - 29 Eriksson JG, Forsén TJ, Mortensen SA, Rohde M. The effect of coenzyme Q10 administration on metabolic control in patients with type 2 diabetes mellitus. *Biofactors* 1999;9(2–4):315–8.
  - 30 Monnier L, Colette C, Owens DR. Glycemic variability: the third component of the dysglycemia in diabetes. Is it important? How to measure it? *J Diabetes Sci Technol* 2008;2(6):1094–1100.
  - 31 Gupta M, Chari S. Lipid peroxidation and antioxidant status in patients with diabetic retinopathy. *Indian J Physiol Pharmacol* 2005;49(2):187–92.
  - 32 Guichardant M, Chantegrel B, Deshayes C, Doutheau A, Moliere P, Lagarde M. Specific markers of lipid peroxidation issued from n-3 and n-6 fatty acids. *Biochem Soc Trans* 2004; 32(1):139–40.
  - 33 Esterbauer H, Schaur RJ, Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med* 1991;11(1):81–128.
  - 34 Njie-Mbye YF, Kulkarni-Chitnis M, Opere CA, Barrett A, Ohia SE. Lipid peroxidation: pathophysiological and pharmacological implications in the eye. *Front Physiol* 2013;4:366.
  - 35 Mancino R, Di Pierro D, Varesi C, Cerulli A, Feraco A, Cedrone C, *et al.* Lipid peroxidation and total antioxidant capacity in vitreous, aqueous humor, and blood samples from patients with diabetic retinopathy. *Mol Vis* 2011;17:1298–304.
  - 36 Izumi N, Nagaoka T, Mori F, Sato E, Takahashi A, Yoshida A. Relation between plasma nitric oxide levels and diabetic retinopathy. *Jpn J Ophthalmol* 2006;50:465–8.
  - 37 Doganay S, Evereklioglu C, Er H, Türköz Y, Sevinç A, Mehmet N, *et al.* Comparison of serum NO, TNF-alpha, IL-1 beta, sIL-2R, IL-6 and IL-8 levels with grades of retinopathy in patients with diabetes mellitus. *Eye* 2002;16:163–70.
  - 38 Castilho Á, Aveleira CA, Leal EC, Simões NF, Fernandes CR, Meirinhos RI, *et al.* Heme oxygenase-1 protects retinal endothelial cells against high glucose- and oxidative/nitrosative stress-induced toxicity. *PLoS One* 2012;7(8):e42428.
  - 39 Leal EC, Manivannan A, Hosoya K, Terasaki T, Cunha-Vaz J, Ambrósio AF, *et al.* Inducible nitric oxide synthase isoform is a key mediator of leukostasis and blood-retinal barrier breakdown in diabetic retinopathy. *Invest Ophthalmol Vis Sci* 2007;48(11): 5257–65.
  - 40 Beyazyıldız E, Cankaya AB, Ergan E, Anayol MA, Ozdamar Y, Sezer S, *et al.* Changes of total antioxidant capacity and total oxidant status of aqueous humor in diabetes patients and correlations with diabetic retinopathy. *Int J Ophthalmol* 2013;6(4):531–6.
  - 41 Valabhji J, McColl AJ, Richmond W, Schachter M, Rubens MB, Elkeles RS. Total antioxidant status and coronary artery calcification in type 1 diabetes. *Diabetes Care* 2001;24(9):1608–13.
  - 42 Astaneie F, Afshari M, Mojtahedi A, Mostafalou S, Zamani MJ, Larijani B, *et al.* Total antioxidant capacity and levels of epidermal growth factor and nitric oxide in blood and saliva of insulin-dependent diabetic patients. *Arch Med Res* 2005;36(4): 376–81.