The antioxidant effect of ubiquinone and combined therapy on mitochondrial function in blood cells in non-proliferative diabetic retinopathy: A randomized, double-blind, phase IIa, placebo-controlled study

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Objectives: To evaluate the effect of ubiquinone and combined antioxidant therapy on mitochondrial function in non-proliferative diabetic retinopathy (NPDR) in a randomized, double-blind, phase IIa, placebo-controlled, clinical trial. Three groups of 20 patients were formed: Group 1, ubiquinone; Group 2, combined therapy; and Group 3, placebo (one daily dose for 6 months).

Methods: Fluidity of the submitochondrial membrane in platelets was determined by examining intensity of fluorescence between the monomer (I_m) and excimer (I_e). Hydrolytic activity of the mitochondrial F_0F_1-ATPase was evaluated with the spectrophotometric method.

Results: Normal, baseline submitochondrial membrane fluidity, 0.24 ± 0.01 I_e/I_m, was significantly diminished in the three study groups vs. normal values (P < 0.0001); placebo, 0.14 ± 0.01 I_e/I_m; ubiquinone, 0.14 ± 0.01 I_e/I_m; and combined therapy, 0.13 ± 0.00 I_e/I_m. Afterward, it increased significantly (P < 0.0001), the ubiquinone group 0.22 ± 0.01 I_e/I_m; combined therapy group, 0.19 ± 0.01 I_e/I_m; with no changes the placebo group. Baseline hydrolytic activity of the F_0F_1-ATPase enzyme increased in the three study groups vs. normal values (184.50 ± 7.84 nmol PO_4), placebo, 304.12 ± 22.83 nmol PO_4 (P < 0.002); ubiquinone, 312.41 ± 25.63 nmol PO_4 (P < 0.009); and combined therapy, 371.28 ± 33.50 nmol PO_4 (P < 0.002). Afterward, a significant decrease the enzymatic activity: ubiquinone, 213.25 ± 14.19 nmol PO_4 (P < 0.001); and combined therapy, 225.55 ± 14.48 nmol PO_4 (P < 0.0001).

Discussion: Mitochondrial dysfunction significantly improved in groups of NPDR patients treated with antioxidants.

Keywords: Diabetes mellitus, Diabetic retinopathy, Antioxidants, Oxidative stress, Co-enzyme Q10, Mitochondrial function

Introduction

Diabetic retinopathy (DR) is a retinal vasculitis caused by complications of diabetes mellitus (DM). Ophthalmic changes that may occur are neovascularization and macular edema. The incidence and prevalence of DR have increased so that it has become the leading cause of visual impairment and blindness in adults in industrialized countries. Sixty percent of patients with a 20-year evolution of type 2 DM present with this vascular microangiopathy. Generally, DR progresses from non-proliferative diabetic retinopathy (NPDR) to proliferative diabetic retinopathy (PDR), which involves the production of reactive oxygen species (ROS). Erythrocytes play an important role in the pathogenesis of DR, especially in the hyperglycemic state, on participating in the production of hemodynamic...
changes with increased blood viscosity, altered permeability, and increased aggregation and adhesion of the erythrocytes to the endothelial cells.\textsuperscript{7,8}

The mitochondria of the retinal cells become dysfunctional on permitting the escape of cytochrome c into the cytosol, with a significant increase in the levels of superoxide (\(O_2^-\)) and an increase in permeability of the mitochondrial transition pores. These alterations unchain a decrease in the activity of the Complex III in the electron transport chain, and limit over-expression of the mitochondrial enzymes that are responsible for trapping the \(O_2^-\); like the manganese superoxide dismutase that catalyzes the reduction of hydrogen peroxide (\(H_2O_2\)) to water and organic peroxides by utilizing glutathione as a reduction source, or by dismutation through the catalytic activity of the catalase.\textsuperscript{9} In the hyperglycemic state, antioxidant levels are diminished and this contributes to the development of oxidative stress. The increase in oxidative stress in the diabetic retina has prompted experimental studies with antioxidant therapies in animal models, which have demonstrated promise. Degradation of the mitochondrial biogenesis in the retina and a decrease in the number of mitochondria, which prevents the transmission of transcription factors of mitochondrial DNA, is present in the hyperglycemic state.\textsuperscript{10} Consequently, the primary, fundamental way to manage prevention of DR is through the strict control of glucose, arterial pressure, and dyslipidemia as therapeutic goals.\textsuperscript{11} In slowing the development of DR mitochondrial biogenesis improves, which could be one potential strategy.\textsuperscript{12,13} Some treatment alternatives include: lipoic acid, vitamins C and E, superoxide dismutase, green tea extract, and some nutritional antioxidant supplements.\textsuperscript{14} However, to date, no medication has been capable of preventing the progression of microvascular damage in DR. Ubiquinone (also known as Coenzyme Q10) is vital for the adequate transfer of electrons in the mitochondrial respiratory chain, and for ATP synthesis.\textsuperscript{15} It is the first line of defense against oxidative damage of the low-density lipoproteins\textsuperscript{16} and could be an antioxidant alternative in patients with NPDR.

Another alternative could be in the combined therapy 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. This combination improves the antioxidant capacity against ROS by protecting the photoreceptors from radiation. Vitamins C and E act in normalizing the numerous biochemical reactions, to slow the aging and degeneration caused by ROS.\textsuperscript{17} Adverse effects of the two aforementioned medications are infrequent. Hence, the objective of the present study was to determine the effects of ubiquinone and combined therapy on mitochondrial function measured in blood cells, in NPDR.

**Materials and methods**

A phase IIa, randomized, double-blind, placebo-controlled, clinical trial was carried out. Sixty subjects with NPDR were selected from 270 patients with DM type 2. Three study groups were formed: Group 1, treated with 400 mg of ubiquinone; Group 2, with combined therapy; and Group 3, with placebo. Pharmacological administration consisted in one 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 reagents (blood donors from the blood bank who agreed to donate an extra 10 ml of blood for the purpose of our study).

The DM, arterial hypertension, and dyslipidemia were managed by the family physicians. Males and females between the ages of 30 and 75 years, with glycated hemoglobin (HbA1c) of \(≤12\%\), were included. The appearance of adverse effects was monitored according to the Official Mexican Guidelines for the Installation and Operation of Pharmacovigilance (Norma Oficial Mexicana NOM-220-SSAI-2012 Instalación y Operación de la Farmacovigilancia); as well as, the Council for International Organizations of Medical Sciences (Consejo de Organizaciones Internacionales de Ciencias Médicas). Adherence to treatment was measured by an independent, external investigator, to calculate the medication ingested by each group, every month. He considered that minimum compliance was 80% ingestion of the medication and dispensed the medications for the following month, throughout the study. Diets, as customized by the patients’ family physicians or nutritionists, were followed. At the study onset and end, the fluidity of the plasma membrane in erythrocytes, the fluidity of the submitochondrial particles of platelets, and the ATP hydrolysis, were evaluated.

**Blood samples**

Peripheral blood samples (10 ml) were obtained from all study participants after an 8-hours overnight fast. Erythrocytes and platelets were separated and stored at \(-80 °C\) until processing. Five milliliters of blood was centrifuged at 310 g for 10 minutes at room temperature, in order to obtain serum.

**Isolation of the erythrocyte membranes and submitochondrial particles in platelets**

Five milliliters (5 ml) of blood was centrifuged with EDTA at 310 g for 10 minutes at room temperature, in order to obtain the plasma and globular concentrate. The plasma was separated and deposited in eppendorf tubes of 1000 μl. The tubes were centrifuged
Fluidity of the submitochondrial membrane in platelets and erythrocyte membranes

Evaluation of membrane fluidity was performed by incorporation of 1,3 diprylenylpropane (DPyP) to the biological membranes. To 35 μl of submitochondrial membranes, 2 ml of Tris–HCl buffer with a pH of 7.8 (10 mM) was added and mixed with a pipette at 24 °C. Using a fluorescent spectrophotometer (Perkin Elmer L550B) the fluorescent intensity of the monomer (I_m) and excimer (I_e) were measured at 395 and 494 nm, respectively. Immediately, 5 μl (0.1 μg) of DPyP was added and then incubated at room temperature, in darkness, for 3 hours, in order to permit the incorporation of the DPyP to the membranes. The second measurement was performed at the same wavelengths and the I_e/I_m ratio of the fluorescence intensity was calculated. The ratio value I_e/I_m is directly related to membrane fluidity. The determination of membrane fluidity in the ghost erythrocytes was performed in the same manner.

ATP hydrolysis

The hydrolytic activity of the mitochondrial F0F1-ATPase was evaluated using the method of spectrophotometry through the liberation of inorganic phosphate, and the results are expressed in nmol PO4. The mitochondria of the platelet particles utilized were isolated through the method described by Baracca et al. To 1 ml of ATPase buffer (125 mM KCl, 40 mM of Mops (pH 8), 3 mM MgCl2), 30 μl of the sample, and 20 μl of ATP (100 mM) were added, and agitated. The tubes were then incubated at 40 °C for 10 minutes to permit the reaction. ATPase activity was stopped with 200 μl of 30% tri-chloroacetic acid. Samples were centrifuged for 10 minutes at 1160 g. To 800 μl of the supernatant, 1 ml of 3.3% ammonium molybdate, and 100 μl of 10% ferrous sulfate were added. Samples were incubated for 20 minutes at room temperature, and the absorbance was read at 660 nm.

Statistical analysis

The Shapiro–Wilks and Mann–Whitney U tests were used to analyze non-parametric data. Results are expressed as mean±standard error. Kruskall–Wallis test (KW) was used to compare the three groups, and the Wilcoxon test was used to compare baseline and final results in each group. Qualitative variables were expressed as frequencies and percentages, and the χ2 test was used. A value of P ≤ 0.05 was considered statistically significant, a power calculation was 80%, and the confidence interval was 95%; Zα = 1.96, alpha value = 0.05; Zβ = 0.84, and beta value = 0.10. All data were computed using an excel database and SPSS PC for Windows version 21 (Chicago, IL, USA) was used for the statistical analysis.

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); and, in ClinicalTrials.gov (Identifier: NCT02062034); in agreement with guidelines as stipulated by the Declaration of Helsinki 1975, as revised in 2000. 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 informed consent and were informed of their results. Patient confidentiality was maintained.

Results

Baseline values were homogenous, comparable, and without selection bias in the three study groups. On comparing averages of the clinical parameters between study groups (KW test), there were no significant differences. Patients had suffered with DM type 2 for >14 years: placebo group, 14.2 ± 1.3; ubiquinone, 15.4 ± 1.6; and combined therapy, 15.2 ± 1.3. Baseline and final glucose results were not significantly different between the study groups: in the placebo group baseline glucose was 125.2 ± 8.0 mg/dL, and final results were 135.3 ± 12.4; in the ubiquinone group baseline glucose was 149.1 ± 12.9, and final 135.7 ± 11.0 mg/dL; and, baseline in the combined therapy group was 149.4 ± 13.5 mg/dL, with a small decrease in the final results to 124.7 ± 10.2 mg/dL. HbA1c
decreased significantly in the placebo group from baseline 9.22% to final 8.01 ± 0.4% (P = 0.048). A similar result was found in the combined therapy group with baseline 9.6 ± 0.4%, and final 8.6 ± 0.2% (P = 0.05). There was no significant difference found for HbA1c in the ubiquinone group, with baseline, 8.5 ± 0.4, and final 8.3 ± 0.4%.

Blood pressure was measured following 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, second, and third measurements was then calculated and registered. The systolic arterial pressure (SAP) was not significantly different between baseline and final measurements in the study groups: placebo group baseline SAP was 128.4 ± 1.3 mmHg, and final 129.5 ± 2.7 mmHg; ubiquinone baseline SAP was 131.4 ± 19.4 mmHg, and final 132.4 ± 3.2 mmHg; and, combined therapy group baseline SAP was 144.5 ± 15.8 mmHg, and final 135.8 ± 3.5 mmHg. The diastolic arterial pressure (DAP) was also not significantly different between baseline and final results in the three study groups: placebo baseline DAP was 74.0 ± 1.1 mmHg, and final 75.1 ± 2.0 mmHg; ubiquinone baseline DAP was 80.7 ± 10.8 mmHg, and final 73.4 ± 2.7 mmHg; and, combined therapy baseline DAP was 84.8 ± 7.2 mmHg, and final 74.9 ± 2.8 mmHg.

**Cellular membrane fluidity in erythrocytes**

Normal fluidity of the ghost erythrocytes was 0.42 ± 0.01 Ie/Im; however, baseline fluidity was significantly diminished in the three study groups (P < 0.001), without significant differences between the groups: placebo group, 0.29 ± 0.01 Ie/Im; ubiquinone group, 0.28 ± 0.01 Ie/Im; and combined therapy group, 0.30 ± 0.01 Ie/Im. At the end of the study, the cellular membrane fluidity of the erythrocytes increased significantly (P < 0.0001) in the ubiquinone group, 0.34 ± 0.01 Ie/Im; and in the combined therapy group, 0.34 ± 0.01 Ie/Im. The placebo group remained unchanged at 0.26 ± 0.01 Ie/Im (see Table 1).

**Fluidity of the submitochondrial particles in platelets**

The normal intensity ratio was 0.24 ± 0.01 Ie/Im. Baseline evaluations prior to pharmacological intervention were significantly diminished vs. normal values in the study groups (P < 0.0001): Placebo, 0.14 ± 0.01 Ie/Im; ubiquinone, 0.14 ± 0.01 Ie/Im; and, combined therapy, 0.13 ± 0.00 Ie/Im. Afterward, fluidity increased significantly (P < 0.0001) in the groups exposed to ubiquinone, 0.22 ± 0.01 Ie/Im; and combined therapy, 0.19 ± 0.01 Ie/Im, respectively. The results obtained in the groups treated with ubiquinone and combined therapy showed a tendency to normalize in the groups treated with antioxidants. A significant increase from baseline values was found in hydrolytic activity of the FoF1-ATPase, which could indicate energy catabolism. End results showed that hydrolytic activity decreased significantly in the groups treated with antioxidants.

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**Table 1 Mitochondrial function**

|                          | Placebo (n=20) | Ubiquinone (n=20) | Combined antioxidant therapy (n=20) |
|--------------------------|---------------|-------------------|------------------------------------|
| Normal value             |               |                   |                                    |
| Erythrocyte membrane fluidity (Ie/Im) | 0.42 ± 0.01   | 0.29 ± 0.01       | 0.29 ± 0.01                        |
| Platelet submitochondrial membrane fluidity (Ie/Im) | 0.24 ± 0.01   | 0.26 ± 0.01       | 0.26 ± 0.01                        |
| Hydrolase of F0-F1-ATPase (nmol PO4) | 184.50 ± 22.83 | 405.50 ± 34.51 | 405.50 ± 34.51                     |

**Redox Report**

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**Cellular membrane fluidity in erythrocytes**

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placebo group did not change, with 0.15 ± 0.01 \( I_e/I_m \) (see Table 1).

**ATP hydrolysis**

An increase in the hydrolytic activity of the baseline \( F_0 F_1 \)-ATPase enzyme vs. normal values (184.50 ± 7.84 nmol PO\(_4\)) was observed in the three study groups: placebo group, 304.12 ± 22.83 nmol PO\(_4\) (\( P < 0.002 \)); ubiquinone, 312.41 ± 25.63 nmol PO\(_4\) (\( P < 0.009 \)); and combined therapy, 371.28 ± 33.50 nmol PO\(_4\) (\( P < 0.002 \)). Hydrolytic activity of the enzyme decreased significantly in the final results in the groups managed with antioxidants: ubiquinone, 213.25 ± 14.19 nmol PO\(_4\) (\( P < 0.001 \)); and combined therapy, 225.55 ± 14.48 nmol PO\(_4\) (\( P < 0.0001 \)), respectively. The placebo group continued in energy catabolism, with 405.50 ± 34.51 nmol PO\(_4\) (see Table 1).

**Discussion**

The increase in ROS provokes changes to temperature and viscosity of the cellular membrane of erythrocytes, with the capacity to affect diapedesis by causing membrane rigidity and decreased blood perfusion. The membrane fluidity of erythrocytes previously reported as normal was 0.305 ± 0.014 \( I_e/I_m \).\(^{19}\) In the present study, fluidity of the cellular membrane of erythrocytes was significantly diminished at the study onset, and increased significantly afterward in the groups treated with ubiquinone and combined therapy. Normal fluidity in the membranes of erythrocytes is of utmost importance to the pathophysiological mechanisms involved in the development and presence of DR, for the production of alterations to blood flow of the retina, and the rheological properties of the blood: with the capacity to cause hypoxia, lower perfusion, and lesser capacity for the erythrocytes to change shape.\(^{122–24}\)

In NPDR, there was significantly lower fluidity in the submitochondrial particles of platelets from the study onset; although this increased significantly after adjunctive treatment in the groups treated with ubiquinone and combined therapy vs. placebo. A similar result was reported previously in patients with Alzheimer’s disease. These authors reported a result of 0.363 ± 0.014 \( I_e/I_m \) as the normal submitochondrial fluidity of platelets.\(^{19}\) Our results suggest that in the hyperglycemic state the ROS exercise deleterious effects on the internal mitochondrial membrane, altering fluidity by increasing membrane rigidity and impeding normal exchange mechanisms.\(^{25}\) The reduction in fluidity of the submitochondrial particles of platelets can also be due to the accumulation of cholesterol in the mitochondria, and cause dysfunction of the organelle by decreasing efficiency of the respiratory chain and increasing levels of ROS, and by diminishing absorption of the mitochondrial mGSH; thus, decreasing the antioxidant defenses.\(^{26}\)

The hydrolytic activity of the mitochondrial \( F_0 F_1 \)-ATPase of platelets in NPDR was found to be significantly increased compared to normal levels. It is evident that the mitochondria are the primary source of cellular energy, and, as a result, are fundamental in the production of energy and in the normal functioning of tissues that are highly dependent on energy metabolism, like in the retina. The ATPase enzyme is composed of a transmembranal section (\( F_0 \)) that pumps protons, and an extramembranal section (\( F_1 \)) where the synthesis or hydrolysis of ATP occurs. In physiological conditions the primary function of ATPase is the activity of synthesis. In this study, we discovered a significant increase in the hydrolytic function of the enzyme vs. normal values in all patients included; and, interestingly, there was a significant decrease after the administration of ubiquinone and combined therapy. Therefore, we can suggest that at the onset of the study the patients were in energy catabolism and at the end their catabolic state diminished and the synthetic capacity of the enzyme improved through the addition of antioxidants. Similar results were reported in Alzheimer’s disease where greater hydrolytic activity was found compared to healthy controls.\(^{27}\)

Owing to the fact that the number of patients with DR continues to grow and therapeutic options are limited, there is a great necessity to develop new strategies for prevention and methods of treatment for DR. The antioxidant effect of ubiquinone has been commonly reported for the complementary treatment of some illnesses like Parkinson’s disease and DM.\(^{28,29}\) Ubiquinone and combined therapy improve mitochondrial homeostasis and diminish energy catabolism. These data suggest that erythrocyte membranes, submitochondrial platelet particles, and the \( F_0 F_1 \)-ATPase were sensitive to antioxidants; therefore, Ubiquinone and combined therapy could represent a potential, economical, safe, attractive, and adjunctive alternative to improve or prevent NPDR.

Study limitations: when this study was conducted it was not possible to obtain reagents for measuring blood levels of ubiquinone and carotenoids. Levels of ubiquinone and the carotenoids may be affected by a number of different causes, not included in the present study.

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**Disclaimer statements**

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Conflicts of interest The authors report no conflicts of interest.

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); 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 1964, as revised in 64th General Assembly, Fortaleza, Brazil, October 2013. The Good Clinical Practices Guide, and the International Conference on Harmonization for Research in Human Beings. Patients signed informed consent and were informed of their results. Patient confidentiality was maintained.

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