# BENEFICIAL EFFECT OF DOCOSAHEXAENOIC ACID AND LUTEIN ON RETINAL STRUCTURAL, METABOLIC AND FUNCTIONAL ABNORMALITIES IN DIABETIC RATS

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<td>Arnal, Emma; Fundación Oftalmológica del Mediterráneo&lt;br&gt;Miranda, María; Universidad CEU Cardenal Herrera, Fisiología, Farmacología &amp; Toxicología&lt;br&gt;Johnsen Soriano, Sílvia; Fundación Oftalmológica del Mediterráneo&lt;br&gt;Alvarez-Nölting, Raquel; Universidad CEU Cardenal Herrera, Fisiología, Farmacología &amp; Toxicología&lt;br&gt;Díaz-Llopis, Manuel; Facultad de Medicina, Cirugía&lt;br&gt;Araiz, Javier; Facultad de Medicina&lt;br&gt;Cervera, Enrique; Hospital General Universitario de Valencia, Oftalmología&lt;br&gt;Bosch-Morell, Francisco; Universidad CEU Cardenal Herrera, Fisiología, Farmacología &amp; Toxicología&lt;br&gt;Romero, Francisco; Universidad CEU Cardenal Herrera, Fisiología, Farmacología &amp; Toxicología</td>
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BENEFICIAL EFFECT OF DOCOSAHEXAENOIC ACID AND LUTEIN ON RETINAL STRUCTURAL, METABOLIC AND FUNCTIONAL ABNORMALITIES IN DIABETIC RATS

Emma Arnal¹, Maria Miranda¹, Siv Johnsen-Soriano², Raquel Alvarez-Nölting², Manuel Díaz-Llopis³, Javier Araiz⁴, Enrique Cervera⁵, Francisco Bosch-Morell¹,², Francisco J. Romero¹,²

¹Dept. Fisiología, Farmacología & Toxicología, Universidad CEU Cardenal Herrera, Valencia, Spain, ²Fundación Oftalmológica del Mediterráneo, Valencia, Spain, ³Dept. Cirugía, Universidad de Valencia, Valencia, Spain, ⁴Dept. Oftalmología, Universidad del Pais Vasco, Bilbao, Spain, ⁵Servicio de Oftalmología, Hospital General Universitario de Valencia, Valencia, Spain

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†Corresponding author: Dr. Francisco J. Romero, Dept. Fisiología, Farmacología & Toxicología, Universidad CEU Cardenal Herrera, Av. Seminario s/n, 46113-Moncada (VALENCIA), Spain. Tf.: +34961369011, fax: +34961395272

E-mail: jromero@uch.ceu.es

Running title: Effect of lutein and DHA in rat diabetic retina
ABSTRACT

PURPOSE: To assess the effect of docosahexanoic acid (DHA) and lutein (both compounds with anti-inflammatory and antioxidant properties) on experimental diabetic retinopathy. METHODS: Male Wistar rats were studied: non-diabetic controls, untreated diabetic controls, and diabetic rats treated DHA and lutein or the combination of DHA + insulin and lutein + insulin for 12 weeks. Oxidative stress and inflammatory markers, apoptosis and functional tests were studied to confirm biochemical and functional changes in retina of diabetic rats. Malondialdehyde (MDA) and glutathione concentrations (GSH) and glutathione peroxidase activity (GPx) were measured as oxidative stress markers. TUNEL assay and caspase-3 immunohistochemistry and electroretinogram were performed. RESULTS: Diabetes increases oxidative stress, nitrotyrosine concentrations and apoptosis in the retina. At 12 weeks after onset of diabetes, total thickness of retinas of diabetic rats was significantly less than that in control rats. Specifically, the thickness of the outer and inner nuclear layers was reduced significantly in diabetic rats and demonstrated a loss of cells in the GCL. These retinal changes were avoided by the administration of insulin, and DHA and lutein alone or in combination with insulin. Impairment of the electroretinogram (b-wave amplitude and latency time) was observed in diabetic rats. DHA and lutein prevented all these changes even under hyperglycemic conditions. CONCLUSIONS: Lutein and DHA are capable of normalizing all the diabetes-induced biochemical, histological and functional modifications. Specially the cell death mechanisms involved deserve further studies, to allow the proposal as potential adjuvant therapies to help prevent vision loss in diabetic patients.

Key words: diabetes, retina, oxidative stress, apoptosis, docosahexanoic acid, lutein
INTRODUCTION

Diabetes mellitus is a heterogeneous metabolic disorder characterised by hyperglycaemia resulting from defective insulin secretion (type 1), resistance to insulin action (type 2) or both. It is often associated with complications, such as cardiovascular disease, kidney failure, retinopathy, as well as peripheral and autonomic neuropathies. The Diabetes Control and Complication Trial (DCCT) reported a relationship between long term glycemic control and diabetic retinopathy development in insulin-dependent patients.\(^1\) The pathogenic link between hyperglycemia and the establishment of diabetic complications is still not known; furthermore, treatment of diabetic retinopathy is hampered by the lack of understanding of its pathogenesis.

Diabetic retinopathy has long been recognized as a vascular disease that develops in most patients, and it was believed that the visual dysfunction that develops in some diabetics was due to the vascular lesions used to characterize the disease. It is becoming increasingly clear that retinal cells are also affected by diabetes, resulting in dysfunction and even degeneration of some of these neurons.\(^2-4\) Barber et al.\(^5\) analyzed retinal specimens from diabetic patients and observed a significantly increase of apoptotic neurons in these retinas than in those of control subjects. Other authors have established that there are more neuronal cells undergoing apoptosis, particularly in the ganglion cell layer (GCL), in retinas of diabetic rats than in control animals.\(^6,7\)

Oxidative stress is closely linked to apoptosis in a variety of cell types. It could induce apoptosis via increasing membrane lipid peroxidation, injury to other macromolecules, or alterations in signal transduction.\(^8\) Recently, Brucklacher et al., examined the permeability of the hemato-retinal barrier, caspase activity, and gene expression after 1 and 3 months of diabetes, using the streptozotocin-induced rat model of diabetes.\(^9\) The specific gene expression changes observed, confirmed in multiple sets
of animals, indicate that proinflammatory, anti-vascular barrier, and neurodegenerative related genes changes, occur associated with increases in apoptosis and vascular permeability. Busik et al. demonstrated an increase in glucose consumption, mitochondrial superoxide production, ERK and JNK phosphorylation, tyrosine phosphorylation, NF-kappaB, and caspase activation in human retinal endothelial cells after exposure of to proinflammatory cytokines, and not after exposure to 25 mmol/l glucose, in contrast to what happens to human retinal pigment epithelial cells and human Müller cells in culture. These findings suggest that in vivo diabetes-related endothelial injury in the retina may be due to glucose-induced cytokine release by other retinal cells and not a direct effect of high glucose.

In view of this, a suggestive treatment of diabetes complications could be a compound with a broad spectrum of actions, including antioxidant, antiinflammatory and/or antiangiogenic effects. The aim of this work was to study the possible beneficial effects of DHA and lutein on retinal structural, metabolic and functional changes in experimental diabetes. Both compounds have antiinflammatory and antioxidant properties, but their effect on retina impairment associated with diabetes mellitus is not well known.

**MATERIALS AND METHODS**

**Experimental design**

Male, Wistar rats were used in the study (at least, 8 rats in each group). Diabetes was induced in animals by a single intraperitoneal injection of STZ (65 mg/kg) in 0.1 M citrate buffer, pH 4.5. Another set of rats, which received only vehicle, served as the control. Fasting blood glucose levels were measured 72 h after STZ injection. Animals having blood glucose levels >200 mg/dL were considered diabetic. Animals were
randomly divided into the following experimental groups: control rats (group C); control animals treated with lutein (0.5 mg/kg orally, group CL); control animals treated with DHA (13.3 mg/kg orally, group CDHA); untreated diabetic rats (group D); diabetic rats treated with insulin (50 mIU/g, subcutaneous, group DI); diabetic rats treated with lutein (0.5 mg/kg orally, group DL); diabetic rats treated with DHA (13.3 mg/kg orally, group DDHA); diabetic rats treated with insulin and lutein (group DIL); diabetic rats treated with insulin and DHA (group DIDHA).

Animals were treated as described for a period of twelve weeks and were housed in groups of two in cages in a temperature- and humidity-controlled room with a 12 h light-dark cycle. All animals had free access to water. Glycemia and body weights (daily) were monitored during the experiment. Animal care and protocols were in accordance with and approved by the Animal Ethics Committee of the Institution and conformed to the ARVO Statement Statement for the Use of Animals in Ophthalmic and Vision Research as well as the Spanish law regulating animal experiments. At the end of week 12, the rats were killed by cervical dislocation. One of the retinas was dissected and fixed, cryoprotected and cryosectioned. Retinas were fixed for 1 h by immersion in 4% paraformaldehyde, washed four times with 3% sucrose and subsequently put overnight for cryoprotection in 25% sucrose, all of which were in Sörensen’s phosphate buffer. Twelve micrometer cross-sections of the retinas were then cut on a cryostat Leica CM 1850 UV and stored at -20 °C until used.

The other eye was dissected and the retina was homogenized in prechilled 0.2 M potassium phosphate buffer, pH 7.0. This homogenate was used to assay GPx activity, GSH, MDA, and protein concentrations. Samples were kept frozen (-80°C) until biochemical assays were performed.
Blood measurements

Heparinized blood samples were taken from rat tails after overnight fasting of the animals. Plasma glucose levels were measured using the Accutrend® Sensor kit (Roche). Glycated hemoglobin was determined in blood samples obtained by heart puncture immediately before killing the animals, using a kit from Byosistems according to the manufacturer’s instructions.

Biochemical assays

MDA, a lipid peroxidation product, concentration was measured by liquid chromatography according to a modification of the method of Richard et al.16 as previously described.17 GPx activity was assayed as reported by Lawrence et al.18 towards hydrogen peroxide. The disappearance of NADPH was followed spectrophotometrically at 340 nm. GSH content was quantified by the method of Reed et al.19 Nitrotyrosine was determined in homogenates of retinas using an Elisa kit from Deltaclon according to manufacturer’s instructions. Protein content was measured by means of the Lowry method.20

Histologic Processing

Sections of retinas including the optic nerve head were stained with hematoxylin and eosin and used for morphologic studies, which included measurements of the thickness of the total retina, the outer nuclear layer and the inner nuclear layer, the number of rows in the ONL and INL and the number of cells in the GCL. The number of cells in the GCL was quantified by counting cells from the temporal to the nasal ora serrata. For each animal analyzed, three separate eye sections were measured.

Additional retinal cryosections were used for TUNEL analysis and studies of active caspase-3. TUNEL assay was performed by an in situ cell death detection kit (Roche Diagnostics, Mannheim, Germany; 11 684 795 001), according to the
manufacturer’s instructions. Immunohistochemical methods were performed on
cryosections for the detection of active caspase-3. Cryosections were permeabilized
with 1% Triton X-100 for 5 min at room temperature, and non-specific binding of the
antibodies was prevented by incubation with 3% fatty acid-free bovine serum albumin
(BSA) supplemented with 0.2% Tween 20 for 1h. Sections were then incubated over
night with the primary antibody rabbit anti-cleaved caspase-3 (Asp 175, Cell Signaling,
Danvers, MA, USA). After incubation sections were washed and incubated with the
secondary antibody (Alexa™ 488 goat anti-rabbit IgG, Invitrogen, Eugene, Oregon,
USA) for 1h at room temperature in the dark.

All measurements were obtained with a microscope and digital camera (Leica
DM 5000 B).

Electrophysiological techniques

Electroretinogram (ERG) was carried out in scotopic conditions and registered
in a McLab software. Latency tome, a and b wave amplitude were measured.

Statistical analysis

The results are presented as mean values ± SEM. Statistical significances were
assessed by ANOVA followed by the Student’s t-test. The level of significance was set
at p < 0.05.

RESULTS

Table 1 shows the body weight, blood glucose and glycated haemoglobin levels
of diabetic and age-matched control rats. After 12 weeks of diabetes induction, diabetic
rats weighed significantly less than control animals. Blood glucose and HbA1c levels
differed significantly between diabetic and control rats, and treatment with lutein or
DHA did not affect these values. Insulin, as expected, recovered body weight, glycemia
and HbA1c values.
Biochemical assays

Oxidative damage was assessed as the concentrations of the lipid peroxidation product MDA, which was increased in rat retina after 12 weeks of diabetes induction (Figure 1A). GPx activity and GSH concentrations decreased in the retina of these animals when compared with those in the retina obtained from age-matched control rats (Figure 1A, 1B and 1C). Administration of lutein or DHA inhibited the diabetes-induced increase in MDA. Similarly, the decreases in GPx activity and GSH levels detected in the diabetic retina were prevented by the administration of lutein or DHA, alone or in combination with insulin. Lutein or DHA did not affect MDA, GPx activity and GSH values in retinas of control animals (data not shown). Nitrotyrosine concentration was measured in all groups (Figure 1D) and it was increased in diabetic rat retinas. Treatment with DHA alone was the only one that could not prevent the increase in nitrotyrosine levels, whereas lutein effectively prevented this increase.

Morphometric Evaluation of Diabetic Retinas

Twelve weeks after onset of diabetes, total retina thickness of diabetic rats was significantly reduced, when compared with control rats (Fig. 2A). Specifically, the outer and inner nuclear layers thicknesses were reduced significantly in diabetic animals (Fig. 2A). Additional measurements were made of the number of cells in the GCL of diabetic and control rats. Ganglion cells were densely packed in retinas of control rats, in contrast to diabetic retinas where a loss of cells in the GCL was observed (Fig. 2B). Moreover, a reduction of the number of cell rows in the ONL and INL was observed in diabetic retinas (Fig. 2B). These changes did not appear in the animals treated with insulin, lutein, or DHA, alone or in combination with insulin. Lutein or DHA did not affect retinal thickness of retinas from control animals (data not shown). A hematoxylin
and eosin–stained section of the retina of a control and a diabetic rat is shown in Figure 3.

**TUNEL Analysis and Detection of Active Caspase-3**

Photomicrographs of retinas processed for the TUNEL assay and detection of active caspase-3 are shown in Figures 4 and 5. Diabetic rat retinas showed more TUNEL- and caspase-3–positive cells in the GCL than control rats. The presence of TUNEL-positive cells was not limited to the GCL, the INL and ONL showed occasionally positive cells (cf. Fig. 4). Interestingly, no caspase-3 positive cells were observed in the ONL. The increase in the number of TUNEL and caspase-3-positive cells of the retina were not observed in diabetic animals treated with lutein or DHA. Lutein or DHA treatment did not increase the number of TUNEL or caspase-3-positive cells of retinas from control animals (data not shown).

**Electrorretinogram**

ERG b-wave amplitude decreased in diabetic animals respect to controls (Table 2). Lutein or DHA alone or in combination with insulin treatment restored ERG b-wave amplitude to control values, as did insulin alone too.

**DISCUSSION**

Lutein and DHA are both independently able to restore the diabetes-induced retinal alterations of MDA and GSH levels, as well as GPx activity (Fig. 1). GSH has, among others, an important role in nutrient metabolism, and regulation of cellular events, including gene expression, apoptosis and cytokine production. Sreekumar et al. demonstrated that incubation of retinal pigment epithelial cells with GSH monoethyl ester caused an 84% decrease in vascular endothelial growth factor -A
secretion. Further evidences suggest that VEGF promotes angiogenesis in diabetic retinopathy, pointing at it as a relevant therapeutic target. In recent years, anti-VEGF agents have emerged as new approaches for the treatment of diabetic retinopathy. Pegaptanib, ranibizumab and bevacizumab are currently available intravitreally administered anti-VEGF agents. This is clearly an invasive procedure associated with potentially serious complications, such as endophthalmitis or retinal detachment, which may be significant for patients requiring serial treatment over many years. Moreover, though delivered within the vitreous, anti-VEGF drugs could pass into the systemic circulation, which could potentially result in hypertension, proteinuria, increased cardiovascular events and impaired wound healing. If restoration of retinal GSH levels with lutein or DHA (cf. Fig 1C), is in fact related with VEGF secretion in diabetic retina, oral lutein or DHA administration are certainly a more secure method to decrease VEGF secretion in diabetic retina.

In agreement with the results herein, previous reports have showed the beneficial effects of lutein and zeaxanthin in the retina of experimental diabetes models in relationship to the oxidative-stress associated metabolic changes. Furthermore, it has been demonstrated, in vitro and in vivo, that consumption of long-chain polyunsaturated fatty acids reduced oxidation and lipid peroxidation. Rotstein et al. exposed cells to a pro-oxidant environment and demonstrated that the addition of DHA to the cultures protected photoreceptors from oxidative stress-induced apoptosis. These authors suggest that DHA might operate preserving mitochondrial membrane structure and function by reducing Bax and increasing bcl-2 expression.

The results in Figure 1D show that nitrotyrosine concentration is increased in diabetic rat retina. Nitrotyrosine has been identified as an indicator of cell damage and inflammation, as well as of the production of nitric oxide. NO can be produced by
several nitric oxide synthase (NOS) isoforms, i.e. endothelial NOS (eNOS), inducible NOS (iNOS), and neuronal NOS (nNOS). Leal et al.\textsuperscript{29} demonstrated recently that iNOS is the isoform with a predominant role in blood-retinal barrier breakdown in diabetic retinopathy. It has been shown that iNOS and VEGF are colocalized in retinas of human subjects with diabetes.\textsuperscript{30} PARP (poly(ADP-ribose) polymerase) upregulates iNOS via nuclear factor-kappa B activation.\textsuperscript{31} Interestingly, lutein, the only compound that is able to decrease nitrotyrosine concentration in diabetic rat retina, in the present experimental model (Figure 1D), was able to decrease nuclear factor-kappa B activation in the retina of alloxan-induced diabetic mice.\textsuperscript{14}

In agreement with other authors,\textsuperscript{7,32} figures 2-5 demonstrate a significant loss of cells in the GCL during diabetes and also that these cells die by apoptosis. The results herein show also that lutein or DHA can restore these alterations of the ganglion cell layer. INL and ONL were significantly thinner in retinas of diabetic rats compared with control animals. Interestingly, TUNEL but not caspase-3 positive cells were observed in the ONL of diabetic animals. It has been recently reported the activation of apoptotic inducing factor (AIF) in the inner segments of photoreceptors, in the inner one-third of the outer plexiform layer, in cells in the inner nuclear layer, in the inner plexiform layer, and in ganglion cells.\textsuperscript{33} Our results may fit with the proposal that in this diabetes model, two forms of cell death can coexist in different retinal cell types: the classical apoptosis with the involvement of aspartate-directed caspases in the GCL, and an apoptosis-like caspase-independent cell death mechanism in the photoreceptors. In this sense, other authors have described the importance of AIF and PARP in photoreceptor death in other retinal degenerations like retinitis pigmentosa.\textsuperscript{34}

Although a proper glycemic control is desirable for preventing the development of diabetic complications, it is indeed not sufficient to prevent them completely. The
ability of lutein or DHA to normalize all the parameters mentioned above, allow us to suggest both compounds as potential adjuvant therapies to help prevent vision loss in diabetic patients. The mechanism by which lutein or DHA exert their retinal protection mechanism deserves future studies.

Declaration of interest - None
REFERENCES


LEGENDS TO FIGURES

TABLE 1. Average weights, blood glucose and HbA1C levels of control and diabetic rats. Data are expressed as the mean ± SD. * p< 0.01 vs all other groups, ** p< 0.01 vs C, *** p< 0.05 vs C, CL, CDHA.

TABLE 2. b-wave amplitude (microV) and latency time of the electrorretinogram of the different groups of animals. Data are expressed as the mean ± SD. (* p< 0.01 vs all other groups, ** p< 0.01 vs C, *** p< 0.05 vs DIL, DIDHA).

FIGURE 1. Effect of DHA and lutein on diabetes-induced oxidative stress in the retina MDA content (1A), GSH concentration (1B), GPx activity (1C) and Nitrotyrosine concentration (1D) of rat retina in the different groups studied, as in Material & Methods. The results are the means ± SD. ( * p <0.05 vs all groups; ** p<0.05 vs DIL; *** p < 0.05 VS DI; ¶ p<0.05 VS DL, DDHA, DIL DIDHA, ¶¶ p <0.05 vs DI, DL, DIL, DIDHA).

FIGURE 2. Hematoxylin and eosin-stained cryosections of retinas from diabetic and control rats were subjected to morphometric analysis at 12 weeks after onset of diabetes. (A) Total retinal thickness, thickness of the outer nuclear layer (ONL) and inner nuclear layer (INL) (B) Number of cells in the ganglion cell layer and number of rows in the ONL and INL. Data are the means ± SE. *Significantly different from control (P < 0.05).

FIGURE 3. Hematoxylin and eosin–stained cryosections of retinas of control (A) and diabetic (B) mice (12 weeks after onset of diabetes). The cells of the GCL are uniformly distributed in the control mouse, whereas there are areas of cellular dropout in the diabetic retina. gcl, ganglion cell layer; inl, inner nuclear layer; onl, outer nuclear layer.
**FIGURE 4.** (A) Fluorescence microscopic detection of TUNEL-positive cells in retinas of the principal groups. (a. control group, b. diabetic group, c. diabetic group treated with lutein, d. diabetic group treated with DHA). Bright fluorescent signal (arrows) indicates TUNEL-positive cells. Blue fluorescent signal: nuclei of all cells stained with 4,6-diamino-2-phenylindole (DAPI). (B) Number of TUNEL-positive cells of the GCL in diabetic and age-matched control rat retinas. (* p <0.05 vs all groups).

**FIGURE 5.** (A) Fluorescence microscopic detection of caspase-positive cells in retinas of the principal groups. (a. control group, b. diabetic group, c. diabetic group treated with lutein, d. diabetic group treated with DHA). Blue fluorescent signal: nuclei of all cells stained with 4,6-diamino-2-phenylindole (DAPI). (B) Number of caspase-positive cells of the GCL in diabetic and age-matched control rat retinas. (* p <0.05 vs all groups).
Table 1.

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Fig. 1

269x190mm (96 x 96 DPI)
Fig. 2

254x190mm (96 x 96 DPI)
Fig. 5

254x190mm (96 x 96 DPI)