Role of Fish Oil on Amelioration of Injured Cornea and Lens of Diabetic and Hypercholesterolemic Male Wistar Rats

Abstract

The association between diabetes and hypercholesterolemia and cataract represents the main public health problem. Little information about the biochemical changes in lens during the progress of disease parallel with lens ultrastructure. Also, the interrelationship between lens and cornea and the dramatic effects of both diseases in cornea was still not clearly illustrated. Our objective was designed to the role of diabetes and hypercholesterolemia in the development of lens and corneal damages and the ameliorated role of fish oil-treatment. Sixty-four male albino rats of Wistar strain were arranged into eight groups (n=8). These are control, fish oil-supplementation, diabetes (single ip streptozotocin of 40 mg/kg body weight in citrate buffer pH 4.6), diabetes and fish oil, hypercholesterolemia (fed on diet rich of 3% cholesterol), hypercholesterolemia and fish oil, diabetes and hypercholesterolemia, diabetes and hypercholesterolemia and fish oil. Treatments were carried out for 16 weeks. Rats were sacrificed and lenses and cornea were separated. Lenses were examined biochemically for glycated protein, endothelin-1, adhesion molecules (ICAM-1 & VCAM-1), zinc and iron parallel with disorganized ball and socket and degeneration of lens fibers. The cornea of both diabetic and/or hypercholesterolemic possessed damage of corneal epithelium, hyalinization and degeneration of both Bowman’s and stromal collagenous layers associated with diffuse leukocytic inflammatory cells in the peripheral collagenous stromal layers and degeneration of endothelium lining the Descemet’s membrane. Hypercholesterolemia showed the least dramatic effect in comparison with diabetes. Fish oil supplementation ameliorated the lens and corneal structure and improved the biochemical changes.

The author finally concluded that the amelioration role of fish oil-supplementation attributed to the reduction of glycated proteins, endothelin-1, adhesion molecules and lens zinc and iron contents which reflected in the structural components of lens fibers as well as improved the elementary structures of cornea.

Keywords: Lens; Cornea; Diabetes; Hypercholesterolemia; Fish oil

Background

Diabetes mellitus is a worldwide disease widespread is in association with obesity; and affected more than 285 million people and expected to increase to 439 million by 2030 [1]. Diabetes and hypercholesterolemia are inter-related with each other associated with the increased oxidative stress and the development of cataract which represent the main integral part in impairing vision affecting 80 million patients and 18 million blindness [2-6].

The cornea is present in front of the lens with characteristic transparencies for light to penetrate and vision of the individual. Diabetes and hypercholesterolemia represent the main cause of disrupted lens metabolism and altering protein configuration causing cataract [3,7-10].

Also, corneal arcus was found to be associated with elevated cholesterol, especially in the young as well as in patients with familial hypercholesterolemia [11,12]. Cholesterol deposition was reported in cornea of rabbits [13]. STZ-diabetic mice possessed a decrease of dendritic cells density in ob/ob mice in comparison with the control [14]. Obese and type I & II diabetic C57Bl/6 mice were found to develop motor and sensory nerve conduction deficits as well as a decrease in sub-epithelial corneal nerves, innervations of the corneal epithelium [15]. Diabetes mellitus was found to impair vision via increasing corneal thickness, inducing corneal edema, and altered epithelial basement membrane and reducing anterior stromal keratocyte, endothelial cell densities and altered intensities of corneal innervations [16-18].

Diabetic-related cataract is contributed mainly to impairment of vision [19]. Diabetic lens possessed abnormal curved lenses with increased lens thickness and decreased lens equivalent refractive index and reduction of lens equatorial diameter [20]. Diabetes was found to increase cell death via disturbed interaction of αβ-crystallin by Bax and caspases [21]. In experimental rat models of types 1 and 2 diabetes, the levels of α-, β- and αA-crystallins (αAC) and γ-crystallins were markedly decrease meanwhile γ-crystallin levels were increased coincides with a depletion of both reduced glutathione and adenosine triphosphate [22,23]. On the other hand, Obese rat possessed increased sorbitol level of their lenses [24]. Increased level of cholesterol oxide was detected in patient with cataractous lenses [25]. Also, Girao et al. confirmed these findings by exposure transparent membranes from clear lenses to free radicals generated 2-amidinopropane and observed the presence of 7 alpha-hydroxycholesterol (6%), 7 beta-hydroxycholesterol (19%), 5 & 6

alpha-epoxycholestanol (1%) and 7-ketocholesterol (74%) [25]. X-ray diffraction of cataractous lenses revealed increased level of cholesterol domains [26]. El-Sayyad et al. reported the presence of cataractous lenses in offspring maternally fed on high cholesterol diet [6].

Fish oil composed of two main inclusions, omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the precursors of eicosanoids (Moghadasian, 2008). In vitro studies carried out by Perales et al. revealed that 5-hydroxycholesterol led to apoptic cell death of corneal smooth muscle cells, initiated by increased level of Bax protein and overexpression of Bax gene. Fish oil-treatment reversed these dramatic changes to the anti-apoptotic pathways [27].

Phytosterol esters was found to have a role in countering hypercholesterolemia-related changes in the brain by decreasing the cholesterol levels, increasing the phospholipid levels and increasing the level of antioxidant enzymes [28]. Fish oil was found to inhibit the production of proinflammatory mediators in rats fed on hypercholesterolemic diet [29].

The present study aims to evaluate the role of Menhaden fish oil in ameliorating the dramatic changes in cornea and lens of diabetic and hypercholesterolemic rats.

Materials and Methods

Chemicals

All of the chemical used were of highest purity. Streptozotocin (N-(Methylnitrocarbamoyl)-α-D-glucosamine), cholesterol (3β-Hydroxy-5-cholestene, 5-Cholen-3β-ol) and Menhaden fish oil were supplied from Sigma-aldrich company (USA).

Experimental work

Sixty-four male albino rats weighing approximately 100 g body weight, obtained from Breading Farm, Ministry of Health, Giza, Egypt. They were fed on standard diet and water was allowed ad libitum throughout the experimental period. The animals were housed in good ventilation with 12 hour light and dark cycle. Male rats were fed on diet containing 3% cholesterol for 16 weeks. Diabetes was carried out and animals allowed feeding on normal healthy diet for the mentioned period treatment. Rats were arranged into eight groups (n=8) such as Control (C), Fish oil-treatment (F) (orally for the mentioned period treatment. The animals were housed in good ventilation with 12 hour light and dark cycle. Male rats were fed on diet containing 3% cholesterol for 16 weeks. Diabetes was carried out and animals allowed feeding on normal healthy diet for the mentioned period treatment. Rats were arranged into eight groups (n=8) such as Control (C), Fish oil-treatment (F) (orally administered every other day at dose 100 mg/kg body weight), hypercholesterolemic-group (H), hypercholesterolemic & fish oil-treatment (HF), diabetic-group (D), diabetic and fish oil-treatment (DF), combined hypercholesterolemic and diabetic group (HD) and combined hypercholesterolemic and diabetic group and fish-oil-treatment (HDF). The animals were maintained on diabetes and / or hypercholesterolemia as well as treatment with fish oil for 16 weeks.

Induction of diabetes

Experimental diabetes mellitus was induced by a single iperteroneal injection of streptozotocin (40 mg/kg) in citrate buffer (0.05 M) (pH 4.5) [30]. Treatment was carried out for 16 weeks. Control animals were treated with physiological saline as vehicle. Hyperglycemia was verified by measuring the blood glucose within the range of 180-220 mg/DL.

Induction of hypercholesterolemia

The experimental group was fed a hypercholesterolemic diet composed of 3% cholesterol and 15% cocoa butter and 0.2% cholic acid and 0.2% thioaracil for 16 wks [31]. The control group was fed on a normal healthy diet free from atherogenic components.

At the end of experiment, male rats of the control and treated groups were sacrificed and ocular regions were removed and dissected for separation cornea and lens and investigated as follows:

Biochemical investigation: (a) Carboxyl & glycation end products: Protein carboxylation was assessed by the reaction of carbonyl groups with 2, 4-dinitrophenyldiazine (DNPH) to form 2, 4-dinitrophenylhydrazones which determined spectrophotometrically at 360-385 nm against standard carbonyl protein. AGE products contain CML, pentosidines and other AGE structures which is determined using ELISA Kit of Cell Biolabs, (Inc., 7758 Arjons Drive, San Diego, CA 92126 USA, Cat.No. STA-317). AGE content in protein samples was determined by comparing its absorbance with that of a known advanced glycation end product and bovine serum albumin (AGE-BSA) standard curve.

(b) Endothelin: Endothelin-1(ET-1) was determined using Endothelin-1 of R&D system (Inc. 614 McKinley Place NE Minneapolis, MN 55413, Cat.No. DET100). The method is based on competitive inhibition reaction between biotin labelled Endothelin1 and unlabelled ET-1 with the pre-coated antibody specific to ET-1. Avidin conjugated with horseradish peroxidase is added and the amount of bounded HRP is proportional to the amount of ET-1 in the lens which is measured by spectrophotometer at 450 nm (within 30 min to avoid fading). Standard curve was plotted using ET-1.

(c) Determination of adhesion molecules (ICAM-1 and VCAM-1): Adhesion molecules were determined by ELISA kit of R&D Systems, Inc (614 McKinley Place NE Minneapolis, MN 55413, USA, Cat. No. DET100). Color development was carried out and the optical density was determined at a wavelength of 450 nm with the wavelength corrected at 620 nm.

(d) Determination of zinc and iron contents: Lens samples of both control and experimental groups, washed thoroughly with distilled water and weighed. They were dried and mixed well by using chloroform methyl mixture 2:1 for lipid extraction. A known weighed of sample per each experimental group was digested by 1 ml of nitric acid at highest purity and diluted with 4 ml bi-distilled water. Zinc and iron were measured by atomic absorption spectrometry [32].

Scanning electron microscopic investigation: Lenses of both control and treated groups was fixed in 2.5% glutaraldehyde (pH 7.4) and dehydrated in ascending grades of ethyl alcohol. The specimens were dried in a carbon dioxide critical point apparatus, mounted in stubs and coated with a thin film of gold at DC sputtering and investigated under scanning electron microscope JOEL5300 JSM (Musashino 3-chome akishima Tokyo 196-8558, Japan).

Histological investigation: Cornea of both control and treated groups were fixed in 10% phosphate buffered formalin (pH 7.4). They were dehydrated in ascending grades of ethyl alcohol, cleared in xylol and mounted in molten paraplast at 58-62 °C. Five micron sections were obtained stained with hematoxylin & eosin and assessed.
histopathological changes under a bright field Olympus microscope.

Transmission electron microscopy: Cornea of the studied animal groups were removed and fixed in 2% buffered glutaraldehyde, dehydrated in ascending grades of propylene oxide and mounted in epoxy resin. Ultrathin-sections were cut and stained with lead citrate and uranyl acetate and examined in Joel transmission electron microscopy 100X (Tokyo 196-8558, Japan).

Statistical analysis: Data were presented as mean±standard error. The statistical analysis was performed with multi-variant analysis of variance (MANOVA) using SPSS (version 13) software package for windows for comparing the multivariations between each specie and p<0.05 was considered statistically significant.

Results
Biochemical assays
Table 1 illustrates lens protein carbonylation, glycation end product, endothelin, adhesion molecules (ICAM-1 & VCAM-1) and zinc and iron contents of diabetes and or hypercholesterolemia and amelioration role of fish oil-supplementation. Comparing with the control, there were marked increase of protein carbonylation (PC) and glycation end product (GEP) of diabetes being 3.57±0.32 and 4.14±0.19 respectively. In hypercholesterolemia, their levels were increased but less than diabetes-treatment being 3.19±0.17 and 3.88±0.18 respectively. However, diabetes and hypercholesterolemia exhibited apparent increase being 3.50±0.22 and 4.29±0.10 respectively. The levels endothelin-1 and adhesion molecules (ICAM-1 & VCAM-1) attained marked increase in diabetic and hypercholesterolemia in comparison with either diabetes or hypercholesterolemia-treatment.

Also, the lens zinc and iron contents were markedly increased in diabetic and or hypercholesterolemia. Fish oil-supplementation possessed ameliorations of the assayed parameters in diabetes and or hypercholesterolemia but were still above the normal values.

Scanning electron microscopic observation of lens
Control or fish oil-supplementation possessed regularly oriented lens fibers with intact fixed ball and socket (Figures 1A and 1B). Diabetes and or hypercholesterolemia exhibited disorganized and loosely attached lens fibers with increased deformation of ball and socket (Figures 1C,1E and 1G). Fish oil-supplementation to diabetes and or hypercholesterolemia improved the induced lens lesions and resorted the structural integrity of lens fibers (Figures 1D,1F and 1H).

Light and TEM observation of cornea
Control and fish-oil supplemented male rats showed normal pattern structure of cornea. Externally, it is formed of normally oriented non-keratinized peripheral stratified epithelium. Its basal columnar or cubical cells are resting on basement membrane. Thin sheath of Bowman’s layer is detected underneath the epithelium. The corneal stroma occupied a large space and composed of regular arrangement of collagen fibrils fenestrated by keratocytes. Transparency of cornea is related to its organized pattern arrangement. At the end of stroma, Descemet’s membrane with underlying endothelium is remarked (Figures 2A and 2B).

Diabetic and/ or hypercholesterolemia possessed marked damage of the cornea characterized by apparent thinning of the corneal epithelium and damaged epithelium with either vacuolated or pyknotic nuclei. Hyalineled and degenerated Bowman’s layer was

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<td>4.14±0.19*</td>
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Each result represents the mean±SE (n=5). *, Significant at P<0.05. C: Control; F: Fish oil; D: Diabetes. Abbreviations: GEP: Glycation End Product; ICAM-1: Intercellular Adhesion Molecule; VCAM-1: Vascular Cell Adhesion Molecule; Zn: Zinc; Fe: Iron.
The integrity of the stromal architecture was disorganized and infiltrated by numerous necrotic foci. The keratocytes lacked normal ordinary structure and degenerated. Multifocal or diffuse leukocyte inflammatory cells were observed especially in the peripheral stromal layers. In case of diabetic and hypercholesterolemic group, there was a detected increase of neovascularization manifested by numerous vacuolated regions outlined internally by flattened cells suspected to be endothelial cells. The corneal endothelium lining the Descemet’s membrane become thickened (Figures 1C, 1E and 1G).

In hypercholesterolemic group, there was a detected vacuolar degeneration of epithelial lining cells, hyalinization of collagenous stroma and Bowman’s layer. Necrotic foci were detected with apparent degeneration of keratocytes (Figure 1E).

In experimental diabetic and/or hypercholesterolemic group supplemented fish oil, there was apparent amelioration especially of the peculiar structure of corneal epithelium. The stroma restored almost the keratocyte arrangement (Figures 1D, 1F and 1H).

At ultrastructural level, the control possessed varying epithelial-structures being flattened in the superficial layers with characteristic microvilli in their peripheral marginal cells. The deep corneal epithelium forms the so-called wing cells, having polygonal shape with ovoid indented nucleus. Their basal lamina appeared folded. Underneath the corneal epithelium, the Bowman’s layer is formed of collagenous fibrils. The stroma possessed regularly oriented parallel fibrils. Keratocytes are dispersed in between the stroma and communicated with each other by their branching processes. Descemet’s membrane is composed of varying degree of collagenous densities and enclosed internally by the endothelial lining layer (Figures 2A-2C).

In experimentally diabetic group, there was a detected damaged epithelial cell, abnormal stroma with pyknotic keratocytes and massive degeneration of deep stromal layer and deteriorated Descemet’s membrane and endothelial lining layer (Figures 2A1-2C1). Fish oil supplementation to diabetic group, restored normal pattern structure of epithelium, stroma and Descemet’s membrane (Figures 2A2-2C2).

Experimental hypercholesterolemic group revealed massive necrosis of epithelial lining cells, disorganized stroma with apparent necrosis of deep layer and degeneration of endothelial layer (Figures 3A-3C). Fish oil-supplementation improved the elementary structure of corneal layers (Figures 3A1-3C1).

In experimental diabetic and hypercholesterolemic group, there was a detected increase of epithelial cells with pyknotic nuclei and massive deterioration of stroma with presence of neovascularization and pyknotic keratocytes. The Descemet’s membrane and their endothelial lining layer appeared hyalinized (Figures 4A-4C). Although, fish oil supplementation to experimental diabetic and hypercholesterolemic group, improved the cytological structure, there was a detected necrotic foci in stroma and less improvement in endothelium (Figures 4A-4C1).

**Discussions**

The observed findings revealed apparent increase of lenticular contents of protein carbonylation and glycation end products. Similar findings were reported by Zarina et al. [33]. Diabetic related cataractous lenses were found to possess apparent increase of carbonylated lens proteins [34]. Increased glycosylation of lens epithelial basement membranes was reported in diabetic patients and adult diabetic rats neonatal streptozotocin induced rat model [35-39]. The elevated levels of AGEs in lens of diabetic rat were markedly correlated with apoptosis of lens epithelium and apparent depletion of epithelial cells within affected lenses [40,41]. Øsnes-Ringen et al. observed DNA strand breaks and increased level of oxidized purines in cataractous lenses [42].

The observed findings detected marked increase of endothelin-1 and adhesion molecules (ICAM-1 & VCAM-1) in diabetic and/or hypercholesterolemic lenses. Endothelin-1 (ET-1) was found to cause inhibition of lens active Na-K transport, keeping water balance for
Promoting lens function. Activation of ET receptors led to marked increase of cytoplasmic calcium concentration in cultured lens epithelial cells, the markers of cataracts [43]. Increased lens calcium content was detected in aged related rat cataract formation [44]. It was found that increase lenticular calcium content led to altered protein configuration through induction of disulfide and dityrosine covalent cross-linking and the formation of peroxynitrite, the markers of cataract [45].

Adhesion molecules are a cell surface glycoprotein, overexpressed in lens epithelium by increased fructose level especially, ICAM-1 [46]. Increased expression of ICAM-1, led to a decrease in lens epithelial proliferation and consequently plays a great role in progression of diabetic cataract [47]. Klein et al. mentioned that ICAM-1 had a great role in lenticular inflammation and developing of aging-related nuclear cataract [48].

The observed findings revealed apparent increase of zinc and iron content in diabetic and/or hypercholesterolemic lens. Similar findings of increased lens zinc and iron content were reported by Dawczynski et al. in human cataractous lenses. Zinc was found to be increased in diabetic and cataractous lenses [49-51]. As we know that the superoxide dismutase (SOD) is dependent upon zinc and copper ions for promoting its activity and scavenging superoxide anion which is important part in oxidative stress. Increased zinc content seemed to be attributed to disrupted lens metabolism. Iron was found to be markedly increased in diabetic lens [52]. Concerning iron, Transferrin and Fe concentrations was markedly at higher level in the intraocular fluids in diseased conditions and tend to be accumulated in lens during ocular inflammation. There are two ways of picking iron by tissues, receptor-mediated endocytosis of diferric transferrin and cell membrane mediated by an oxido-reductase [53].

The observed increase of biochemical markers were confirmed by the degeneration, disrupted orientation of lens fibers and deformation of ball and socket in diabetic or hypercholesterolemic rats. Fish oil-supplementation improved the marked increase of carbonylated and glycated lens proteins, adhesion molecules and zinc and iron contents.

Weikel et al. reported that cataractous lenses can be reduced by feeding on diets rich in omega-3 fatty acids in diabetic mice [7]. Also, dietary intakes of omega-3 polyunsaturated fatty acids may resolve the incidence of nuclear cataract in patients [54]. The ameliorating role of fish oil may be attributed to its antioxidant property and presence of fat-soluble vitamins (A, D and E), polyunsaturated fatty acids, sterols and mild amounts of beta carotene as mentioned by Luterotti et al. in cod liver oil [55].

Concerning cornea, which is present in front of lens and
Figure 3: Transmission electron micrograph of cornea of male rats. A-C. Control showing normal epithelium (A) regular stroma with keratocytes (B) and Descemet’s membrane (C) A1-C1. Diabetes showing damaged epithelium with distorted basal lamina (A1), damaged keratocytes within stroma (B1) and degenerated of both stroma and Descemet’s membrane (C1) Arrow head showed folded basal lamina. A2-C2. Diabetic and fish oil-treatment showing partial improvement. Abbreviation: BM: Basement membrane; BL: Basal lamina; DM: Descemet’s membrane; DDM: degenerated Descemet’s membrane; DK: Degenerated keratocytes; Ep: Epithelium; K: keratocytes; St: Stroma; Ma: Macrophage.

have important role in protection of lens and preserving visual activity. Histo-cytological observations of diabetes and or hypercholesterolemia group revealed apparent damage of corneal epithelium with either vacuolated cytoplasm or pyknotic nuclei. There was a detected hyalinization and degeneration of both Bowman’s and stromal collagenous layers as well as of stromal keratocytes. Multifocal or diffuse leukocytic inflammatory cells aggregation was detected in the superficial collagenous stromal layers. In case of diabetic and hypercholesterolemic group, there was a detected increase of neovascularization explained by numerous spaced regions outlined internally by flattened cells suspected endothelial cells as well as degeneration of the endothelium lining the Descemet’s membrane (DM). Hypercholesterolemic showed the least damage comparing with diabetes and or hypercholesterolemia (Figures 2-5)

Similar findings were reported in cornea of 24 & 30 M old rats [56].

Diabetic patients, KKAy mice, rat and monkey exhibited pronounced alterations in corneal epithelium, fragility of collagenous stroma and degeneration of keratocytes [57-60]. Friend et al. mentioned that diabetic rats possessed apparent degeneration of basal epithelial cells associated with thickening and folding of their basement membrane leading to increase sorbitol pathway products. Altered endothelium may interfere with the maintaining transparency of stroma as a result of increased edematous lesions as detected by hyalinization and disrupted vision [61-63].

In experimental diabetic and or hypercholesterolemic group supplemented fish oil, there was apparent amelioration especially of the peculiar structure of corneal epithelium. The stroma restored almost the keratocyte arrangement. In vitro cultures of both cornea endothelial and lens epithelial cells with 10 mg/ml cholestarol revealed apparent increase of apoptosis and DNA fragmentation associated with over expression of interleukin-1b-converting enzyme and CPP32

Figure 4: Transmission electron micrographs of cornea of male rats. A-C. Hypercholesterolemic showing vacuolar degenerated and pyknotic epithelium (A & Aa), distorted stroma with damaged keratocytes (B) and degenerated Descemet’s membrane (C) Arrow showing degenerated epithelium. A1-C1. Hypercholesterolemia and fish oil-treatment showing partial improvement.

Abbreviation; BM: Basement Membrane; DM: Descemet’s Membrane; DDM: Degenerated Descemet’s Membrane; DK: Degenerated Keratocytes; DEP: Degenerated Epithelium; DST: Degenerated Stroma; EP: Epithelium; K: Keratocytes; ST: Stroma; Ma: Macrophage.

Figure 5: Transmission electron micrographs of cornea of male rats. A-C. Diabetes and hypercholesterolemia showing degenerated and pyknotic epithelium (A) degenerated collagenous stroma and keratocytes (B) and damaged Descemet’s membrane and ruptured endothelium (C) A1-C1. Diabetes and hypercholesterolemia and fish oil-treatment showing partial improvement. Arrow head indicated folded basal lamina of epithelial layer and slight vacuolation of collagenous stroma.

Abbreviations: BM: Basement Membrane; DM: Descemet’s Membrane; DDM: Degenerated Descemet’s Membrane; DK: Degenerated Keratocytes; DEP: Degenerated Epithelium; DST: Degenerated Stroma; EP: Epithelium; HD: Hypercholesterolemia and Diabetes; HDF: Hypercholesterolemia and Diabetes and Fish oil; K: Keratocytes; PN: Pyknotic Nuclei; ST: Stroma.
proteases [64]. Mitochondria represent the main integral part of corneal lens epithelium. Damage of lens epithelium in contributed to the mitochondrial damage and consequently increased the liberation of free radicals and intern affected the corneal region [65].

Fish oil supplementation was found to enhanced healing of corneal damage induced by scopolamine as well as photoreactive keratotomy and improve corneal innervation in streptozotocin-treated mice [66-68].

The authors finally concluded that diabetes and or hypercholesterolemia interfered with degeneration and disruption of lens fibers assessed by increase lenticular biomarkers and associated with corneal damage. Fish oil supplementations scavenge free radicals and ameliorated both lens and cornea.

References


