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Research Article

Influence of Selected Micronutrients on Glycation of Human Lens Proteins: Implications in Diabetic Cataract

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Abstract

Lens crystallin's are long-lived proteins and undergo age related glycation leading to several complications including cataract. Human lens is among the several other tissues wherein the formation of dicarbonyl compounds such as advanced glycation end-products (AGEs) occurs. In the present part of the study, selective micronutrients viz. ascorbic acid, α-tocopherol, β-carotene, retinol, riboflavin, thiamin, folic acid, niacin, pyridoxine, zinc, iron, manganese and selenium were investigated for their possible effect on methyl glyoxal (MGO) mediated, *in vitro* glycation and formation of protein carbonyls. The soluble fraction of human lens protein was used as the substrate. Fluorescence was measured as formation of AGEs at the wavelength pair of 370 and 440 nm. Quercetin, aminoguanidine, tannic acid and gallic acid were used as standard antiglycation agents. A decline in the formation of AGEs and protein carbonyls was observed with α-tocopherol, riboflavin, zinc and manganese, which could be ascribed to their antioxidant and free radical scavenging activity.

Keywords: Cataract; Methyl Glyoxal; Diabetes; Non-Enzymic Glycation; Advanced Glycation End-Products

Introduction

Age related cataracts are the most significant cause of blindness across the globe. Efforts to lower the cataract burden will be of great social and health economic benefit [1,2]. Glycation is one of the major events compromising the lens transparency through decrease in the chaperone activity of crystallin's. The long-lived lens proteins are highly susceptible to post-translational modifications such as glycation. The formation of Advanced Glycation End Products (AGEs) is a result of excessive nonenzymatic glycation of lens proteins that could further lead to opacification and hence cataract [3-7]. Many aldehydes and ketones, in addition to sugars, are known to form AGEs. Methylglyoxal and glyoxal have been identified as a major source for AGE formation in several tissues including the lens. Methylglyoxal is known to be formed nonenzymic ally by amine-catalyzed sugar fragmentation reactions and by spontaneous decomposition of triose phosphate intermediates in glycolysis. It is also a product of the metabolism of acetal, an intermediate in the catabolism of both threonine and the ketone body acetone [8]. It has been reported that methylglyoxal binds and modifies a number of proteins, including BSA, RNase A, collagen, lysozyme and lens crystallin's [9,10]. Methyl glyoxal is known to induce formation of heterogeneous AGE and protein cross-linking. Moreover, the nature of AGE structures and extent of modifications depend largely on the glycating reagents and conditions employed for the in vitro glycation reaction [11-16]. *In vivo* and recent human clinical studies have served to underscore the pathophysiological importance of AGEs [17].

Current research into the AGE pathway is proceeding along various fronts. Several potential drug candidates as AGE inhibitors have been documented. The health beneficial properties particularly the antioxidant activities of essential micronutrients have been extensively studied [18-21]. *In vitro* and *in vivo* studies have shown promising results for pyridoxamine and ascorbic acid [22- 24] for

prevention of protein glycation. Aminoguanidine is the first drug investigated both *in vitro* and *in vivo* [23]. Despite the large amount of information available on the pathological significance of AGE protein in cataract genesis, data on the effect of micronutrients on AGE formation as well as the dicarbonyl modification is scanty. This study was undertaken to screen 9 vitamins and 5 trace metals for their *ex vivo* effect on MGO mediated glycation using the soluble fraction of human lens protein.

Methods and Materials

This study did not involve any invasive procedure. All the enrolled patients who had undergone cataract surgery were the lens donors and participation in the study was purely on voluntary basis with a written informed consent. Protocols followed throughout the study were in accordance with 'Ethical Guidelines for Biomedical Research on Human Subjects' of 'Indian Council of Medical Research', New Delhi, India, ICMR (2000) and are in accordance with the declaration of Helsinki.

Chemicals

All the chemicals and reagents used for the present study were of AR grade. Retinol, β-carotene and α-tocopherol, methyl glyoxal and aminoguanidine were procured from Sigma Aldrich Chemicals, USA; Gallic acid, tannic acid, riboflavin, ascorbic acid, thiamin, folic acid, niacin, pyridoxine, zinc, copper, iron, manganese and selenium were procured from Hi-Media Co., India.

Preparation of samples

Lens protein glycation

Prior to cataract surgery, all lenses were graded for opacities using a slit lamp biomicroscope after pupillary dilation by the ophthalmologist, using the Lens Opacification Classification System (LOCS III). Lenses with nuclear opalescence grade 1.0 and 2.0 were selected for the study whereas those of grade 3.0 or more were excluded from the study. Nuclear portions of lens were obtained from individuals undergoing routine extra capsular lens extraction. The age of the lens donors did not exceed 62 years to avoid interference of other age related complications. The extracted lens as homogenates were collected from the ophthalmologist immediately after the surgery and preserved at -20°C for further biochemical analysis. The soluble part of the protein rich fraction was separated. After suitable dilution to bring each of the homogenate to a constant opacity, these were subjected to MEG mediated glycation alone or in presence of micronutrients.

The method used for the initiation of glycation was as previously described [7]. Briefly, the soluble protein fraction of the lens was separated under ice-cold conditions, and incubated with a quick glycating agent, methyl glyoxal (5 mM MGO), in phosphate buffer containing 0.05% sodium azide, (pH 7.4) at 37°C under sterile conditions. The standard glycation inhibitors used were quercetin, aminoguanidine, tannic acid and gallic acid. At the end of 72 hours, the samples were removed and extensively dialyzed for removal of any unbound protein remnants or sugars. The physiological concentrations of all the 13 micronutrients were considered while selecting their levels and on the basis of reported studies, a 10 fold higher level (10X) than their normal blood levels was chosen for initial screening.

Estimation of AGEs

The relative degree of glycation was assessed by measuring intrinsic fluorescent signals from AGEs using a Fluorescence Spectrophotometer (F 2500, Hitachi, Japan). Emission scans from 400 to 550 nm (slit, 10 nm) with an excitation wavelength of 370 nm (slit, 10 nm) were taken to assess the overall effect of micronutrients on levels of AGEs formation. The results were expressed in terms of arbitrary units (AU)/mg protein. The protein concentrations were determined using Lowry's method [25].

Estimation of protein carbonyls

Protein carbonyl groups were estimated by the method of Uchida., *et al* [10]. Briefly, 0.5 ml protein samples were mixed with an equal volume of 0.1% solution of 2, 4-Dinitro Phenyl Hydrazine (DNPH) in 2M HCl and incubated at room temperature for 1h. After incubation, protein was precipitated by 20% Trichloro Acetic acid (0.5 ml) and washed three times with 1ml of ethanol/ethyl acetate (1:1) mixture. Later, the precipitate was solubilized in urea (6M) and absorbance was read at 365 nm on spectrophotometer (Thermo Electron Corporation, UV1). Concentration of protein carbonyls was calculated by using molar extinction coefficient ($\varepsilon 365$ nm = 21 mM^{-1} cm^{-1}). The results were expressed as nM carbonyls/mg protein. The protein content was determined by the Lowry method using BSA as standard.

Statistical analysis

All the samples were analyzed in triplicates. Mean and standard deviation were calculated for all the samples. Single factor as well as two way analysis of variance (ANOVA) was used to evaluate the differences in response of micronutrients and glycation inhibitors. Statistical significance was defined at $p < 0.05$ and $p < 0.01$.

Results

Lens glycation

Initially, to check the variability in the glycation response of MGO, a pilot experiment comprising of 5 sets of lens homogenates (in triplicates) each representing a different human lens donor was undertaken. Figure 1 shows the MGO glycation response of lenses by different donors. Further, four standard glycation inhibitors viz. quercetin, aminoguanidine, gallic acid and tannic acid (1% w/v, aqueous) were chosen as reference antiglycation agents for the validation of experimental protocol. ANOVA indicated significant decrease in glycation for all the four compounds as compared to MGO control for lens ($F = 96.1$, $p < 0.001$). Multiple comparison test for glycation indicated that among the inhibitors tested, quercetin was the most potent antiglycation agent, followed by aminoguanidine, gallic acid and tannic acid (Figure 2).

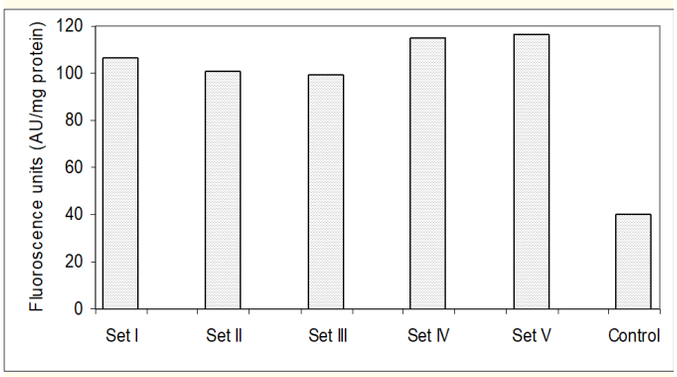


Figure 1: Response of MGO glycation by varying lens donors. (Each observation is an average of triplicate experiments)

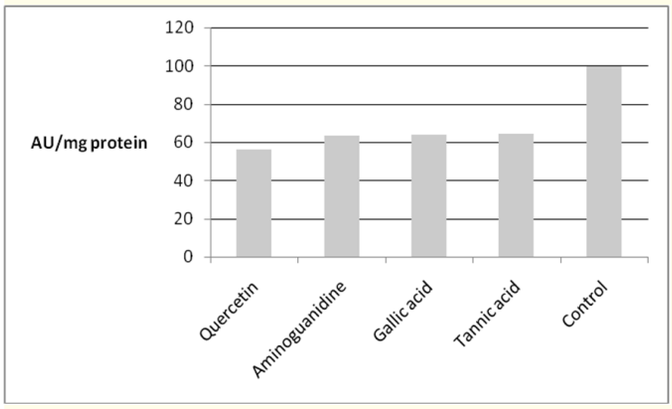


Figure 2: MGO glycation in presence of standard glycation inhibitors.

The decline in AGE fluorescence in presence of different vitamins and trace metals has been shown in figures 3 and 4 respectively. All the micronutrients showed significant inhibitory effects on glycation except folic acid which showed only a marginal non-significant decrease ($p > 0.05$). Further, α-tocopherol followed by riboflavin showed prominent inhibitory response. Zinc and manganese showed higher inhibitory activity in the trace metal subgroup. Fluorescence spectra were recorded at ex 370 nm and emission 380 - 800 nm to confirm the behavior of promising micronutrients. Figure 5 shows the fluorescence spectra for zinc and manganese that indicates gradual decrease compared to the positive control. Furthermore, a dose response experiment for AGE fluorescence was undertaken for the selected micronutrients as shown in figure 6. It was noted that manganese and riboflavin inhibited glycation at a very low concentrations as compared to zinc and vitamin C.

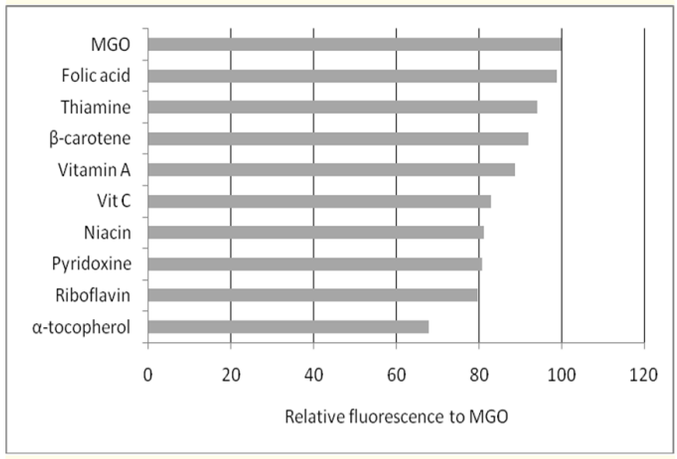


Figure 4: Effect of trace metals on glycation of MGO.

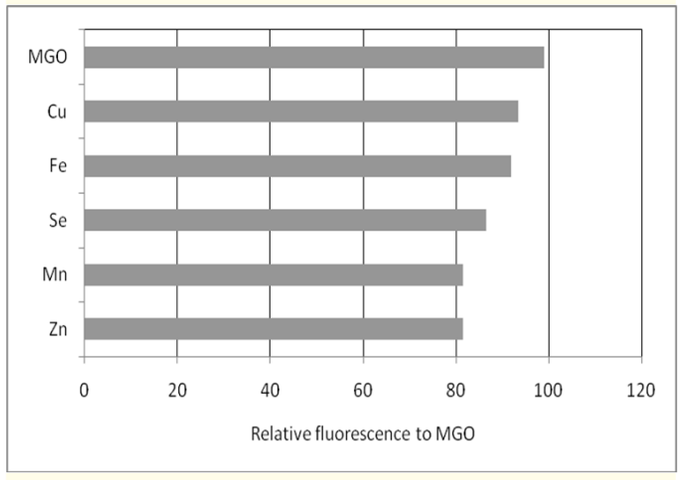
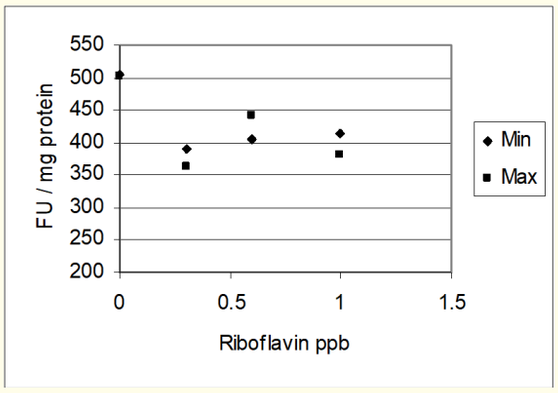
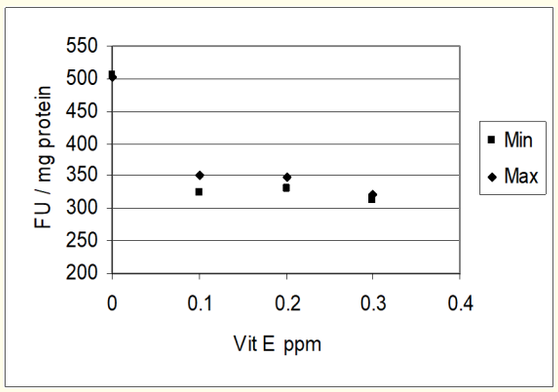
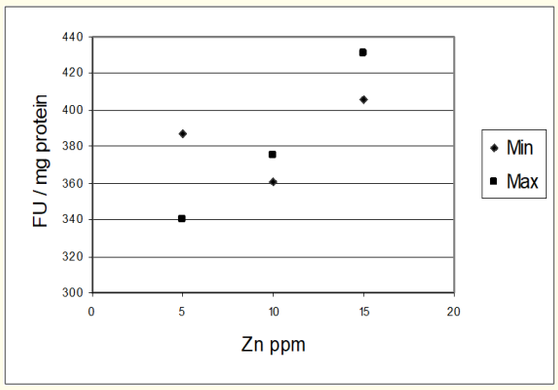


Figure 4: Effect of trace metals on glycation of MGO.







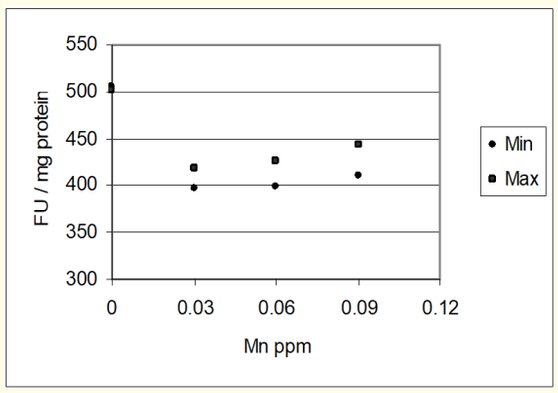


Figure 5: Dose response of 4 micronutrients during lens glycation.

ppb: parts per billion - micrograms per Litre.

ppm: parts per million - milligrams per Litre.

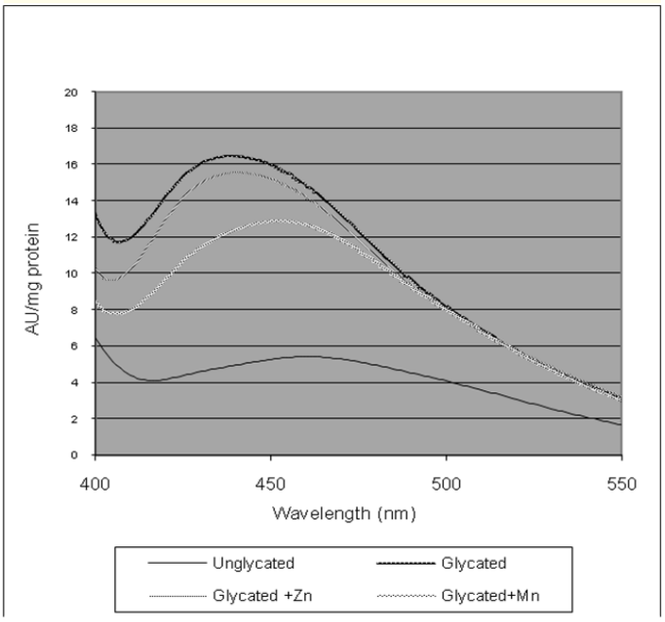


Figure 6: Fluorescence spectra of glycated lens proteins in presence of zinc and manganese.

Formation of protein carbonyls

As AGEs measurement is nonspecific, the carbonyl estimations by DNPH assay are is considered a valuable biomarker to assess the extent of protein oxidation in the lens. It was observed that all the screened micronutrients elicited lower levels of carbonyls with varying response compared to positive control. The most promising micronutrients were α-tocopherol, riboflavin, zinc and manganese that showed potential decrease. Figure 7 shows the responses seen for the screened micronutrients in decreasing the formation of carbonyls compared to the positive control.

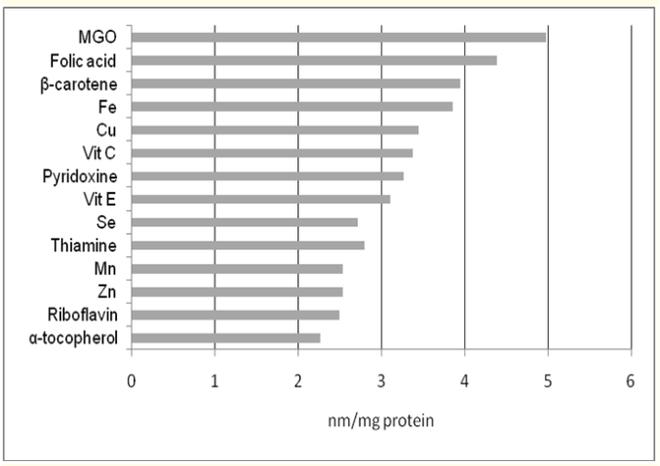


Figure 7: Levels of protein carbonyls in presence of micronutrients.

Discussion and Conclusion

The lens contains structural proteins (α-, β-, and γ-crystallin's), which are long lived and undergo a variety of post-translational modifications like glycation, carbonylation, steroid adducts formation, protein-protein disulfide bond formation, methionine oxidation, racemization, degradation, and deamidation [26,27]. These modifications, singly or in combination, cause changes in crystallin's that lead to their aggregation and cross-linking during aging. The ultimate result of the changes is protein solubilization due to aggregation and cross-linking and cataract development.

It was preferred to make use of the human lens protein as model protein due to its longevity and susceptibility to extensive accumulation of AGE, which is enhanced in diabetes and associated pathologies.

For screening large number of candidate micronutrients as possible antiglycation factors, it was felt necessary to have a quick glycation initiator that could readily glycate proteins. Methylglyoxal (2-oxopropanal) is one such reactive α-oxoaldehyde and physiological metabolite of the glyoxalase system. The rate of formation of methylglyoxal in human red blood cells under euglycemic conditions is approximately 125 pg/day, which increases in hyperglycemia and by the addition of fructose, D-glyceraldehyde, dihydroxyacetone, acetone, and hydroxyacetone [8]. In one of the reported studies, the median concentration of MGO increased 5-6-fold, relative to control values in blood samples of patients with insulin-dependent diabetes mellitus and 2-3-fold in patients with non-insulin-dependent diabetic mellitus [9]. The pathological significance of this was indicated in model that showed a link between methylglyoxal metabolism and the development of retinopathy, neuropathy, and nephropathy. Besides, in foodstuffs and beverages, MGO is formed during processing, cooking, and prolonged storage. Fasting and metabolic disorders and/or defects in MGO detoxification processes cause accumulation of this reactive dicarbonyl *in vivo*. In addition, the intake of low doses of MGO over a prolonged period of time can cause degenerative changes in different tissues. This background prompted us to use MGO for the present experiment.

Oxidative stress has also been shown to play a role in the pathophysiology of intraocular inflammatory diseases [13,14]. In one of the reported studies using rapid glycation response [10], AGEs in cataractous lens homogenates of patients who underwent phacoemulsification was estimated through *in vitro* protein model of glycoxidation. Analysis of 30 cataractous lenses (15 diabetic and 15 non-diabetic) revealed a significant increase in both glycated lens proteins of diabetics compared with the controls ($P < 0.01$) and AGE - linked fluorescence at 440 nm ($p < 0.01$). The reported antiglycation actions of riboflavin and α-tocopherol and proglycating action of iron are confirmed in the present results. Folic acid on the other hand was a poor inhibitor. Moreover, the glycation inhibitory influences of zinc and manganese have emerged as a new finding which needs further studies for confirmation.

Although various mechanisms have been proposed to explain the pathophysiology of diabetic complications, oxidative stress remains to be the major determinant [28]. Hence, agents or compounds which have a potency to show multiple actions such as antidiabetics/antiglycating having good antioxidant potential could be more effective. Therefore, we selected the antioxidant micronutrients as target molecules for evaluating glycation as formation of AGEs and dicarbonyls. There is also substantial evidence that oxidative stress is at least partly responsible for galactosemic cataract, and that antioxidants can be effective inhibitors of cataract genesis [29,30].

Although there are numerous studies with regard to the pathological significance of AGE proteins in cataract genesis, relatively little is known about the effect of AGE formation in lens proteins, particularly dicarbonyl modification [7-9].

In-vitro and in-vivo studies have shown that the action of ROS on proteins results in the formation of carbonyl groups. Carbonyl groups (aldehyde and ketone) can be introduced into proteins by a variety of oxidation reactions [31,32]. Protein peroxidation, in contrast to lipid peroxidation, does not have the features of a chain reaction. The oxidized proteins are selectively removed by proteinases and, according to some investigators, could also partly accumulate in cells. Increase of oxidatively modified proteins is associated with decrease in proteasome activity in aging epidermal cells. Human studies related to protein oxidation use protein carbonyl content as marker for oxidative damage. The plasma proteins damaged by peroxidation have a long half-life. Therefore, evaluation of carbonyl group content in plasma proteins provides a significant clue to the severity of oxidative stress under disease conditions [14]. Carbonyl formation in proteins is dependent on metal ions such as Fe^{+2} and $Cu^{+2.}$ These can bind to the cation binding site in proteins and with help of H_2O_2 or O_2 they change the side chains of amino acids to carbonyl groups. By involving in the Fenton reaction, Fe^{+2} and Cu^{+2} catalyze the production of hydroxyl radical, which oxidizes lipids, proteins and DNA [11,12]. These oxidation products accumulate in pathological conditions such as cataract.

Present *in vitro* model had therefore incorporated estimation of protein carbonyls.

Micronutrients as possible agents for AGE inhibition

All of the micronutrients studied are naturally occurring in the body and several of them such as ascorbate are known to be lowered in the tissues of subjects with diabetes [20,21]. In our study, the decrease in carbonyls has been shown by zinc and manganese. This may be due to the fact that iron and copper have two oxidation states and act as pro oxidants while zinc having single oxidation state acts as antioxidant. The inhibitory action of α-tocopherol on carbonyl formation might also be due to its action as a potent radical scavenger. These results need to be further confirmed.

A major breakthrough in cataract research would be to find a therapeutic means of slowing down the protein glycation [33,34]. Our previous studies on '*in vitro*' albumin glycation reported promising potential for Trolox (α-tocopherol analogue), riboflavin, zinc and manganese [35,36]. The present part of the study was undertaken in the same line to further confirm selected vitamins and trace elements for their role in protein carbonyls and AGE formation using the lens protein fraction. The beneficial effects of some of the micronutrients like manganese, riboflavin and α-tocopherol provide an indication for their potential utility as possible therapeutic agents in inhibiting glycation and management of diabetic cataract.

This fact and the results of the present study point to the necessity of good nutrition as well as a possibility of inexpensive strategy for the prevention of diabetic complications like cataract [37]. The importance of antioxidant micronutrients and their possible therapeutic use for the maintenance of ocular health nevertheless beget further studies to confirm their protective role.

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