



ROLE OF LUTEIN IN INHIBITING ENDOGENOUS OXIDATIVE DNA DAMAGE IN LYMPHOCYTES WITH THE OCCURRENCE OF AGE-RELATED MACULAR DEGENERATION

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ABSTRACT

AMD occurring from damage to the retinal pigment epithelial (RPE) cells thought to be mainly caused by oxidative stress. The present investigation is focused on to evaluate oxidative damage to the DNA in association with AMD in isolated human peripheral lymphocytes and its inhibition by Lutein. Sunflower (*Helianthus annuus* L.) petals were used as source to isolate Lutein and the uptake of Lutein by lymphocytes was assessed by HPLC analysis of Lutein in the cell pellet. The DNA damage induced by H₂O₂ was assessed by agarose gel electrophoresis and comet assay. The HPLC analysis of Lutein from the cell pellet showed the presence of Lutein and Zeaxanthin (retention time of 10.3 and 20.8min) in comparison with all-*trans*-isomers of Lutein and Zeaxanthin standards at retention times of 10.5 and 19.8min respectively. Lutein was able to protect DNA damage at an effective concentration of 1:1 and also at 1:2M/M (molar/molar) ratio of H₂O₂:Lutein. Similar results were obtained when single cell gel electrophoresis was done. H₂O₂ produced 57 comets/100 cells, which on treatment with Lutein at 1:1 and 1:2.0M/M ratio showed only 11 and 10 comets/100 cells respectively. There was four folds decrease in the comet formation when compared to H₂O₂ treated cells. The results indicate that Lutein act as potent antioxidant in preventing oxidative DNA damage in relation to AMD thus play a significant role in modulating the pathogenesis of AMD.

Keywords: Lutein, Zeaxanthin, *Helianthus annuus* L, Oxidative stress, Age-related Macular Degeneration, DNA damage.

INTRODUCTION

Age-related macular degeneration (AMD) is one of the most common causes of severe visual loss in the elderly population in the developed world^{1,2}. The prevalence of AMD varies from 1.2% to 29.3%^{3,4}. Three population based studies-the Beaver Dam Eye Study⁵, Blue Mountain Eye Study⁶, and the Rotterdam Study⁷ report the prevalence rates to be 1.7% in US, 1.4% in Australia and 1.2% in Netherlands respectively. The prevalence varies from 1.1% in South India⁸ to 4.7% in North India⁹. Oxidative stress has been implicated in the pathogenesis of AMD. In the tissues, the impairment of retinal pigment epithelium (RPE) cell function is an early and crucial event in the molecular pathways leading to AMD^{10,11}. It has been projected that by the year 2020, approximately about 2.95 million people will have advanced AMD¹². Clinically relevant AMD results from damage to the RPE cells thought to be mainly caused by oxidative stress. The stress also affects the DNA of RPE cells, which promotes genome instability in these cells. These effects may coincide with the decrease in the efficacy of DNA repair with age. Therefore individuals with DNA repair impaired more than average for a given age may be more susceptible to AMD if oxidative stress affects their RPE cells.

The RPE cells function in an environment that is rich in endogenous reactive oxygen species (ROS). The activity of RPE cells, the high local oxygen concentration, and the exposure to light contribute to the production of ROS. Although multiple physiologic mechanisms protect the RPE from the toxic effects of light and oxidative damage,

mounting evidence suggests that chronic exposure to oxidative stress over the long term may damage the RPE and predispose it to the development of AMD^{13,14}.

The carotenoids are the most widespread group of pigments in nature, with over 600 characterized structurally. They are present in all photosynthetic organisms and responsible for most of the yellow to red colours of fruits and flowers. Lutein is one of the carotenoids, found in many fruits and vegetables including mangoes, corn, sweet potatoes, carrots, tomatoes and dark leafy greens. The cell culture, animal and human studies has been directed to the potential role of Lutein and Zeaxanthin in protecting against several chronic diseases, particularly age-related macular degeneration (AMD) and cataract, cancer at various sites, heart disease and stroke¹⁵.

The macula of the eye contains two carotenoids, Lutein and Zeaxanthin¹⁶ with Zeaxanthin being the dominant component in the central macula and Lutein distributed throughout the retina. There is an inverse relation between macular pigment density and lens density, suggesting that the macular pigment may serve as a marker for xanthophylls in the lens¹⁷. Possible biologic mechanisms of the protective role of Lutein and Zeaxanthin include their ability to filter harmful shortwave blue light and function as antioxidants. Their antioxidative properties provide a solid basis for investigating their potential in slowing the development of age-related macular degeneration (AMD), in which oxidative damage is believed to play a role in the pathophysiology.



Helianthus annuus L. (Sunflower, Asteraceae) has been an important resource of natural oil and lipid-rich nutrients for centuries. It is widely used in human foods because of its highest protein concentrate in the seed flour. Additionally, it is used as a preventive medicine against diuresis, diarrhea, and several inflammatory diseases¹⁸. Recently, it was shown that aqueous extract of sunflower seed inhibits cell damage induced by hydrogen peroxide or amyloid β -peptide in SH-SY5Y neuroblastoma cells¹⁹. The plant is well known for its allelopathic compounds, several phenol and terpene compounds²⁰. Leaves of *Helianthus annuus* L. possess antimicrobial activity against many bacteria²¹. The plant extracts is shown to heal inflammation during experimental wound. In this context, the present investigations are aimed at checking the level of DNA damage measured with the alkaline comet assay induced by hydrogen peroxide in isolated human peripheral blood lymphocytes in relation to AMD and its inhibition by Lutein isolated from *Helianthus annuus* L. petals.

MATERIALS AND METHODS

Plant material and chemicals

Petals of *Helianthus annuus* L. were collected from authentic vendors in the local market of Mysore, Karnataka. The petals were washed in double distilled water to remove any extraneous material and air dried in an open space under aseptic condition for about 3-5 days and ground to fine powder, sieved through 100mesh. The powder was stored in air tight polypropylene bottle at room temperature (RT). Lutein standard was obtained as kind gift from Dr. ML Shankar Narayan, Cancor India Ltd. Cochin, Kerala. All the chemicals and solvents used were of analytical grade from Sisco Research Laboratories (SRL), India.

Isolation and spectral analysis of Lutein from *Helianthus annuus* L. petal extract

Isolation of Lutein from *Helianthus annuus* L. petal powder and spectral analysis of Lutein standard (10 μ g/20 μ l) and Lutein fraction was done according to the procedure described in our previous publication²².

Human peripheral lymphocyte isolation and culture

The peripheral lymphocytes were isolated from 10 to 15ml of freshly drawn venous blood from healthy male donors aged between 25–30 years. Blood was collected in anticoagulant Acid Citrate Dextrose (ACD, 85mM citric acid, 71mM trisodium citrate, 165mM D-glucose) in the ratio of 5:1. To this four volumes of hemolysing buffer (0.85% NH₄Cl in 10mM Tris buffer, pH7.4) was added, mixed well and incubated at 4°C for 30min. Then the cells were centrifuged at 1200rpm for 12min, the supernatant was discarded, pellet was washed again in 5ml of hemolysing buffer and the cell pellet was washed thrice with 10ml of Hank's Balanced Salt Solution (HBSS, 137mM NaCl, 5mM KCl, 0.8mM MgSO₄ and 5mM D-glucose in 8.5mM phosphate buffer pH7.4) and suspended in the

same buffer solution. Cells were suspended in RPMI–1640 media supplemented with 12% fetal calf serum supplemented with glutamine. Temperature was maintained at 37°C in a humidified 5% CO₂ incubator²³. The medium was changed at an interval of 24hrs. The viability of lymphocytes was determined by trypan blue dye exclusion method²⁴. The percent viability of cells was always more than 96%, in each batch of cells isolated.

Uptake of Lutein by human peripheral lymphocytes

Preparation and composition of synthetic micelles was based on the literature described earlier^{25,26}. Aliquots of stock solutions of phosphatidylcholine (PC) in chloroform and all-*trans*-Lutein in ethanol were combined in a 30 ml glass vial. The mixture was vortexed at room temperature for 30 min. The clear solution was filtered sterilized (0.22 μ m pores). To characterize uptake of micellarized Lutein, spent medium was removed and cells were washed twice with RPMI-1640 medium at 37°C. Cells were collected in 1.5 ml ice-cold phosphate buffered saline (pH 7.2, 10mM), centrifuged (1,800 rpm, 4°C, 5 min) and cell pellet was stored at –20°C for a maximum one week. Lutein was quantified in all samples within one week.

Lutein was extracted from cell pellet by addition of 1 ml of petroleum ether:acetone (2:1). Samples were vortexed and centrifuged as above to facilitate separation of phases. The extraction was repeated a total of three times. Combined petroleum ether fractions were dried at room temperature, resolubilized in methanol and quantified by high performance liquid chromatography (HPLC) according to the method described earlier²⁷ with slight modifications. HPLC (Hewlett Packard model HP 1050) with UV/Visible detector set at 450 nm was used for analysis. Lutein was separated using a C30 analytical scale (4.5 x 250 mm) reversed phase column with a C18 stationary-phase guard column. Separation was achieved using a gradient elution with methanol:ammonium acetate (98:2). The concentration of Lutein was calculated from comparison of area under the curve with known concentrations of the all-*trans*-isomers of Lutein and Zeaxanthin standards at retention times of 10.5 and 19.8 respectively.

DNA isolation and agarose gel electrophoresis

Lymphocytes DNA was isolated according to the method reported earlier²⁸. Cells were washed thoroughly with Hank's Balanced Salt Solution (HBSS, 137mM NaCl, 5mM KCl, 0.8mM MgSO₄ and 5mM D-glucose in 8.5mM phosphate buffer pH7.4) and the cells were homogenized with homogenizing buffer (0.3M Tris buffer, pH8.0 containing 0.2M sucrose, 0.1M NaCl and 0.01M EDTA). To this, 125 μ l of 10% SDS was added, vortexed and the mixture was incubated at 65°C for 30min. The contents were cooled and 350 μ l of 8M potassium acetate was added and kept on ice bath for 60min. The contents were spun down at 5000rpm for 20min at 4°C and the supernatant was transferred to a fresh eppendorf tube. The DNA was extracted by Tris saturated phenol:chloroform (1:1) mixture. The contents were



mixed well and centrifuged at 3000rpm for 10min at 4°C. DNA was precipitated from the organic phase by adding equal volume of ice cold ethanol and dissolved in 100µl of 1X TBE buffer (10mM Tris, 2mM Boric acid and 1mM EDTA). Final concentration of DNA was calculated by using the following formula, DNA (µg/ml)=Final dilution X Optical density at 260nm / 20 X Volume of the sample.

DNA samples were run on 1% agarose gel prepared in Tris-Boric acid-EDTA (TBE) buffer (pH 8.0, 10mM) and the ethidium bromide was incorporated into the gel at a concentration of 10µg/ml. 2µg of DNA was loaded on to the wells and run in TBE buffer, pH8.0 at 60V for 60min. Hind III digest of λ-phage DNA were used as molecular size markers. The bands were visualized and photographed under ultra violet light transillumination.

Single cell gel electrophoresis assay

DNA strand breakage was determined in exponentially growing human peripheral lymphocytes. After the desired incubation period with or without Lutein (20µg/ml) at 37°C for 120min, the medium was aspirated off and cells were rinsed with EBSS (Earle's Balanced Salt Solution). Cells were then resuspended in fresh medium and the cell density was calculated by counting number of cells in a haemocytometer. The cells were then centrifuged at 1500rpm for 5min at 4°C. The supernatant was then quickly removed and to the pellet 1ml of Minimal Essential Medium (MEM) supplemented with 20% Fetal Calf Serum (FCS) was added. 200µl of this suspension was then added to 1ml of ice cooled phosphate buffer saline, 10mM, pH7.4 and left for 5min. This mixture was then centrifuged at 1500rpm for 5min at 4°C. Cells were then resuspended in 1% (w/v) Low Melting Point (LMP) agarose and poured on to a microscopic glass slide pre-coated with 1% (w/v) Normal Melting Point (NMP) agarose.

The slides were then placed in ice cold lysis solution (0.85% NH₄Cl in 10mM Tris buffer, pH10) and left overnight at 4°C. Slides were then placed in an electrophoresis tank covered with electrophoresis buffer (1mM NaOH, 0.1mM EDTA, pH13) at 4°C for 40min to allow DNA unwinding before electrophoresis. After the desired period, electrophoresis was carried out at 30V for 45min at 4°C. Slides were then placed in neutralizing buffer (0.4M Tris, pH7.4) at 4°C for 5min. This step was repeated thrice. Then the slides were dried at 37°C before scoring of comets. After drying, 50µl of LMP was added to each gel to allow hardening and 20µl of ethidium bromide was added at a final concentration of 10µg/ml.

Cells were scored for DNA damage visually. An average of one hundred comets was scored per slide based on the fluorescence in the comet tail²⁹.

Statistical analysis

Statistical analysis was done in SPSS (Windows Version 10.0.1 Software Inc. New York) using a one-sided students *t*-test. All the values represent mean of

triplicates and are expressed as Mean±SD. *p*<0.01 and *p*<0.05 was considered as significant.

RESULTS AND DISCUSSION

AMD is an ocular disease leading to loss of central vision in the elderly people. The disease is characterized by both primary and secondary damage of macular RPE cells resulting in formation of drusen, choroidal neovascularization and atrophy of photoreceptors and choriocapillaris layer of the choroidea. AMD has been linked to poor dietary habits; primary prevention is based on a healthy and balanced diet. Phytochemicals, such as antioxidants and anti-inflammatory agents, help prevent or delay the progression of many pathological conditions. Observational and clinical trials have been conducted to support the safety of higher intakes of the phytochemicals such as Lutein and Zeaxanthin and their association in reducing the risks of cataracts and improving clinical features of AMD. Additional phytochemicals of emerging interest, like green tea catechins, anthocyanins, resveratrol, and Ginkgo biloba, shown to ameliorate ocular oxidative stress, hence deserve greater attention in clinical trials³⁰.

There are reports which determine the effect of food supplements or eating foods rich in antioxidants that can protect against age-related macular degeneration, a disease in which the central portion of the retina deteriorates so that only peripheral vision remains. A team of Australian researchers who followed 3,654 subjects of age 49 or older found no statistically significant association between AMD and dietary intake of carotene, zinc, or vitamins A or C, either from diet, supplements or both. Other published studies have had conflicting results, with some finding correlations and others finding none³¹.

Lutein is a yellow colored pigment. Although Lutein is not categorized as a vitamin, dietary Lutein is believed to be an essential nutrient for normal vision. Lutein is fat soluble, a deficiency may occur if fat digestion is impaired. Lutein is found in egg yolk and in many plants and vegetables, including red peppers, mustard, broccoli, zucchini, corn, garden peas, spinach, leek, collard greens and kale. Lutein is responsible for the coloring of many fruits and vegetables. Lutein is an antioxidant which is believed to be an essential nutrient for normal vision³². Studies have also indicated that Lutein improves heart health, protects our skin against UV damage, reduces diabetes induced oxidative stress, and possesses anti-inflammatory and anticancer properties³³. The central part of the retina, called the macula, contains macular pigments in which Lutein is concentrated. The yellow colored pigments protect the retina from damage of the photo-oxidative affect of high-energy radiations. Lutein offers eye protection by lowering the risk of age related vision loss, which causes gradual loss of central vision³⁴. Age related vision loss or age-related macular degeneration is caused by steady damage of the retina.



In the present study, Lutein was isolated from sunflower (*Helianthus annuus* L.) petals. Sunflower seeds and oil is shown to serve as nutraceutical against many pathological conditions. In this context, petals were selected for isolating Lutein because they are usually dumped as waste after harvesting the seeds. These petals are rich source of colored compounds. The spectral analysis of hexane extract of sunflower showed the presence of Lutein when compared with Lutein standard with a signature wavelength at 421nm, 446nm and 472nm (data not shown). The uptake of Lutein by lymphocytes was assessed by determining the concentration of Lutein in the cells. The HPLC analysis of Lutein from the cell pellet showed the presence of Lutein and Zeaxanthin (retention time of 10.3 and 20.8min) in comparison with all-*trans*-isomers of Lutein and Zeaxanthin standards at retention times of 10.5 and 19.8min respectively (Figure 1a and 1b).

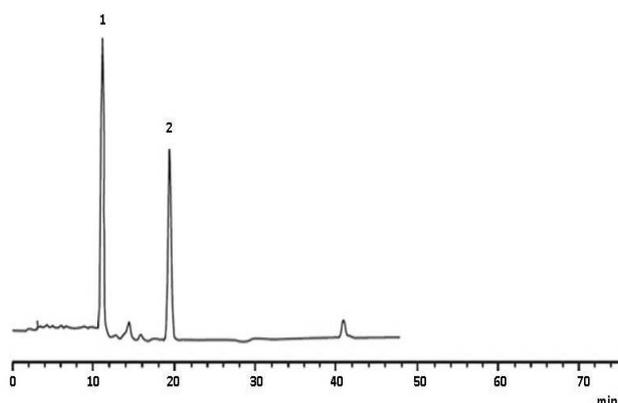


Figure 1a: HPLC of all-*trans*-isomers of Lutein and Zeaxanthin standards

Lutein (15 μ g/50 μ l) and Zeaxanthin (20 μ g/50 μ l) was quantified by HPLC (Hewlett Packard model HP 1050) with UV/visible detector set at 450 nm on C30 analytical scale (4.5 x 250 mm) reversed phase column with a C18 stationary-phase guard column. Separation was achieved using a gradient elution with methanol:ammonium acetate (98:2). The concentration was calculated from the area under the curve of the all-*trans*-isomers of Lutein (**Peak 1**) and Zeaxanthin (**Peak 2**) standards at retention times of 10.5 and 19.8 respectively.

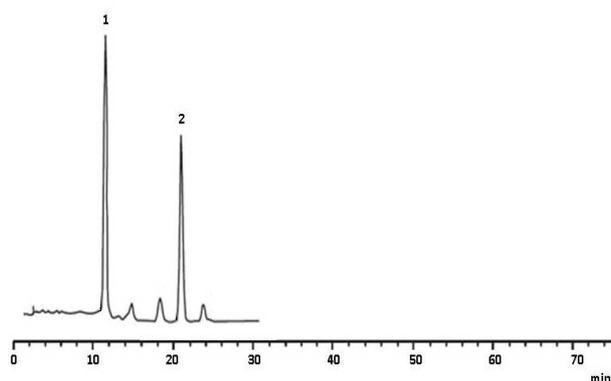


Figure 1b: HPLC of Lutein and Zeaxanthin isolated from lymphocytes

Lutein (35 μ g/50 μ l) and Zeaxanthin (45 μ g/50 μ l) was extracted from cell pellet by addition of 1 ml of petroleum ether:acetone (2:1) and quantified by HPLC (Hewlett Packard model HP 1050) with UV/visible detector set at 450 nm generally was used for analysis. Lutein was separated using a C30 analytical scale (4.5 x 250 mm) reversed phase column with a C18 stationary-phase guard column. Separation was

achieved using a gradient elution with methanol:ammonium acetate (98:2). The concentration of Lutein (**Peak 1-retention time of 10.5min**) and Zeaxanthin (**Peak 2-retention time of 20.8min**) was calculated from comparison of area under the curve with all-*trans*-isomers of Lutein and Zeaxanthin standards.

The uptake of Lutein and Zeaxanthin in human eyes is remarkably specific because many other prominent serum carotenoids such as α -carotene, β -carotene, β -cryptoxanthin, and lycopene have not been found in eye tissues. Lutein and Zeaxanthin are structural isomers. They are polar than many other carotenoids and have hydroxyl groups on the cyclic end rings. The hydroxyl groups may allow them to incorporate into the cell membrane in an orientation that stabilizes the membrane. The only structural distinction between Lutein and Zeaxanthin is the placement of one double bond. The allylic hydroxyl of Lutein is much more easily oxidized than the secondary hydroxyl groups present in Zeaxanthin^{35,36}. It is likely that specific transport or binding proteins are involved. However, this needs to be further proved by showing specificity which is currently in progress in our laboratory.

The cells of the human body are continuously attacked by Reactive Oxygen Species (ROS), which arise as natural byproducts of normal cellular energy production or are generated in large amounts by exhaustive exercise or by chemical agents in the environment³⁷. The reactivity of free radicals varies from relatively low, as in the case of oxygen molecule itself, to a very high, as in the case of the short lived and highly reactive hydroxyl radical (\cdot OH). Fatty acids are susceptible to attack by ROS such as hydrogen peroxide (H₂O₂), superoxide (O₂⁻), peroxy radicals (R-OO \cdot), singlet oxygen (¹O₂) and in particular hydroxyl radical (\cdot OH), hence any reaction which forms ROS would surely stimulate lipid peroxidation. Hydrogen abstraction is easier in unsaturated fatty acids than their saturated fatty acids.

Cells have evolved a complex network of defense barriers to counteract the generation of ROS and protect against the oxidation of macromolecules by scavenging ROS. The dietary intake of antioxidants is thought to play a major role in this network. The antioxidant capacity of fruit and vegetables plays an important role in their protective effect^{38,39}. An approach to investigate antioxidant effects is to use DNA as the biological target molecule; two methods appear to be the tests of choice: measurement of 7-hydroxy-8-oxo-2'-deoxyguanosine (8-oxodG) and the comet assay. From a mechanistic perspective, dietary antioxidants are expected to inhibit the formation of oxidative DNA damage. When ROS are formed *in vitro* or by oxidative stress *in vivo*, several types of oxidative DNA lesions are formed, including small base lesions and exocyclic adducts⁴⁰.

In the present paper we checked the correlation between the levels of DNA damage measured with the alkaline comet assay in relation to AMD. This technique is a versatile and sensitive method for measuring DNA damage such as single and double strand breaks as well

as alkali-labile sites in DNA. Most oxidative damage associated with AMD will occur within post mitotic cells of the retina and will be environmental in origin. Peripheral blood lymphocytes were chosen as model cell system as they would be affected by the environmental condition causing oxidative DNA damage in the retina. Oxidative DNA damage was detected in lymphocytes because most studies assessed damage in this cell population. They should have been performed on the retina cells, but these cannot be obtained from live AMD patients as easily as the lymphocytes. Lymphocytes are easily accessible and their genetic constitution with the regards of DNA repair processes reflects that of the retina cells⁴¹. In our study, the lymphocytes were isolated from normal healthy volunteers and then exposed to H₂O₂ to assess the oxidative damage to cellular DNA and its inhibition by Lutein.

DNA isolated from human peripheral lymphocytes on treatment with H₂O₂ showed complete breakdown into low molecular mass fragment. Whereas, cells when treated with Lutein did not show any significant breakdown of DNA rather it was comparable with the DNA isolated from untreated cells (Figure 2).

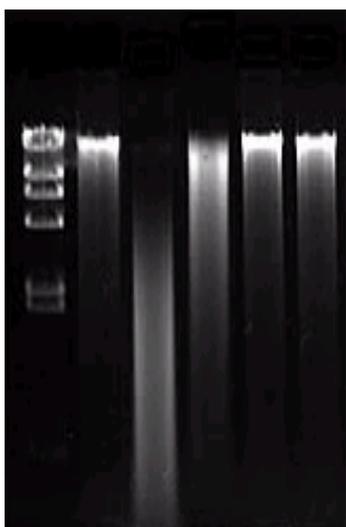


Figure 2: Protective effect of Lutein on H₂O₂ induced DNA damage in lymphocytes assessed by agarose gel electrophoresis

Lymphocytes (1x10⁶ cells) ± Lutein in 0.5ml n-hexane, incubated at 37°C, 20min, added H₂O₂ (1mM), final volume made up to 1ml in n-hexane, incubated 37°C, 1hr. Reaction arrested by keeping in ice cooled bath. 2µg of DNA from cells loaded on 0.8% agarose gel and electrophoresis done [M. Hind III digest of λ-phage DNA molecular marker 1. DNA isolated from untreated lymphocytes (1x10⁶ cells) 2. DNA isolated from H₂O₂ (1mM) treated lymphocytes 3. As in 2 + Lutein (H₂O₂:Lutein, 1:0.5M/M) 4. As in 2 + Lutein (H₂O₂:Lutein, 1:1.0M/M) 5. As in 2 + Lutein (H₂O₂:Lutein, 1:2.0M/M)] as mentioned in materials and methods, M/M (molar/molar).

Lutein was able to protect DNA damage at an effective concentration of 1:1 and also at 1:2M/M (molar/molar) ratio of H₂O₂:Lutein. Similar results were obtained when single cell gel electrophoresis was done. H₂O₂ produced 57 comets/100 cells, which on treatment with Lutein at 1:1 and 1:2.0M/M ratio showed only 11 and 10 comets/100 cells respectively. There was four folds

decrease in the comet formation when compared to H₂O₂ treated cells (Table 1).

Table 1: Protective effect of Lutein against H₂O₂ induced DNA damage as assessed by Single Cell Gel Electrophoresis

Treatment	Number of comets /100 cells
1. Untreated lymphocytes (1X10 ⁶)	4 ± 0.68
2. DNA isolated from H ₂ O ₂ (1mM) treated lymphocytes	57 ± 1.56 [⊗]
3. As in 2 + Lutein (H ₂ O ₂ :Lutein, 1:0.5M/M)	45 ± 1.35
4. As in 2 + Lutein (H ₂ O ₂ :Lutein, 1:1.0M/M)	11 ± 0.91 [*]
5. As in 2 + Lutein (H ₂ O ₂ :Lutein, 1:2.0M/M)	10 ± 0.87 [*]

* Statistically significant at $p < 0.05$ when compared to the group with H₂O₂ alone

⊗ Statistically significant at $p < 0.01$ when compared to the untreated group M/M (molar/molar)

The mechanism of inhibition of DNA damage could be attributed to the potent antioxidant activity of Lutein. Our earlier studies on the ability of Lutein to act as potent antioxidant showed that Lutein at 20µg/ml effectively inhibited peroxidation of lipids, hydroxyl radical formation and DPPH radical formation to the tune of 86%, 92% and 90% respectively, whereas, α-tocopherol, curcumin and butylated hydroxy anisole, when used at dose ~12 times more (400µM) than Lutein showed 75–95% inhibition of lipid peroxidation and scavenging of hydroxyl and DPPH radicals²². However, a detailed investigation on the cell membrane and DNA damage caused by H₂O₂ as a consequence of cellular disruptions and the signaling molecules involved in these processes in relation to AMD is of prime importance in understanding the mode of inhibition by Lutein. In Conclusion our study showed that Lutein act as potent antioxidant in preventing oxidative DNA damage in relation to AMD thus play a significant role in modulating the pathogenesis of AMD.

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