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Antioxidant effect of lycopene on retinal pigment epithelial (RPE) cell line

Sheriff Sheik Abdulazeez[1]*, Balasubramanian Sundaram[1] , Ponmurugan Ponnusamy[2]
[1]College of Applied Medical Sciences, Shaqra University, (KINGDOM OF SAUDI ARABIA)
[2]K. S. Rangasamy College of Technology, Thiruchengode, Tamil Nadu, (INDIA)
E-mail: biosherif@yahoo.co.in
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ABSTRACT

Age-related macular degeneration (AMD) is a common cause of blindness among elderly people and has become greater public health concern around the globe. Epidemiological reports suggest that consumption of large quantity of fruits and vegetables of high carotenoid content have a decreased risk of AMD. Lycopene is potent antioxidant and a carotenoid family member of known health benefits. Thus the present investigation was designed to evaluate the antioxidant property of lycopene on D407 retinal pigment epithelial (RPE) cell lines through lycopene incorporation studies and 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay. The results suggest that lycopene can be a potential candidate to halt the progression of AMD due to its effective defense against oxidative stress and non toxic nature on the RPE Cells.

KEYWORDS

Oxidative stress;
Lycopene;
Ocular damage;
Carotenoids.

INTRODUCTION

Age-related macular degeneration (AMD) is a common cause of blindness among elderly people and has become greater public health concern of developed as well as developing countries. AMD affects about 14 million people worldwide[1]. AMD ranks third among the global causes of visual impairment and causes blindness in 8.7% of the world's population[2]. The clinical features of AMD include drusen, hyperplasia of RPE, geographic atrophy, and choroidal new vessels (CNVs)[3,4]. These Pathologic changes affect retinal pigment epithelial (RPE) cells, Bruch's membrane and the choriocapillaris[5]. Although huge number of studies has been carried out on AMD, the exact pathogenesis of this disease remains unclear. However, oxidative damage by reactive oxygen species (ROS) on RPE found to be a predominant factor for AMD. These ROS are detoxified by endogenous enzymatic and non enzymatic antioxidants of the RPE. Among the non enzymatic antioxidants, carotenoids (β-Carotene, lycopene and lutein etc) plays a vital role, as they are efficient singlet oxygen quenchers and as scavengers of reactive oxygen intermediates[6].

Carotenoids are a class of more than 600

natural pigments that are present in fruits and vegetables[7]. Many investigations around the globe revealed an inverse relationship between the intake of fruits and vegetables and the risk of several types of cancer[8]. Epidemiological reports suggest that consumption of large quantity of fruits and vegetables of high carotenoid content have a decreased risk of AMD[9-13]. Similarly, supplementation of β-carotene along with other antioxidants resulted in a significant reduction in the development of advanced AMD[14]. Lycopene is a potent antioxidant and member of the carotenoid family. It is the naturally occurring compound that gives the characteristic red color to the tomato, watermelon, pink grapefruit, orange, and apricot. A number of studies have indicated the health benefits of consuming lycopene[15-20]. The identification of a diverse range of carotenoids including lycopene in ocular tissues, its ability to reduce the pathogenesis of diabetes[21] and above all its high oxygen-quenching capacity[22] makes lycopene as a potential candidate to explore its role in AMD. Thus the present investigation was designed to evaluate the antioxidant property of lycopene on D407 RPE cell lines through lycopene incorporation studies and MTT cytotoxicity assay.

EXPERIMENTAL

Chemicals

D407 RPE cells were procured from American Type Culture Collection (Rockville,MD). Cell culture materials and accessories were purchased from GIBCO, USA. Lycopene, Tween 40, antibiotics and all other chemicals were of analytical grade obtained from Sigma Chemical Company (St. Louis, MO).

Cell culture

D407 RPE cells were grown in high glucose Dulbecco's modified Eagle's medium (DMEM) containing sodium pyruvate (1 mM), penicillin (100 U/ml), streptomycin (100 mg/ml) and amphotericin (100 mg/ml) and supplemented with 10% fetal calf serum. Cells were grown in 25 cm^2 culture flasks for incorporation experiment and in 96-well culture plates for other parameters, under 5% CO_2/95% moist air atmosphere at 37° C.

Lycopene solution

Lycopene (1 mmol/L) stock was prepared in Tween 40 in the dark room just before use. Different amounts of lycopene stock solution (1 mmol/L) were added to culture tubes, resulting in final lycopene concentrations of 5 μM/L, 10 μM/ L, and 15 μM/L. The highest amounts of Tween 40 used to dissolve lycopene were added to separate cultures to provide control populations of cells.

Lycopene incorporation studies

To evaluate the effect of lycopene on viability, the cells were grown in 25cm^2 culture flasks until they reach the confluency of 70-80%. At this subconfluency, medium was removed and cells were treated with increasing concentrations of lycopene (5μM, 10μM, and 15μM) in DMEM using a Tween40 vehicle. After different time periods (3, 6, 12 and 24 hours) of incubation with lycopene, the cells were collected by trypsination with 0.05% trypsin in phosphate buffer saline (PBS), washed 3 times with phosphate buffered saline by vortex motion, centrifuged and PBS was removed. After the final wash the cell pellet was treated with 2% cold Triton X100 solution and subjected to lycopene extraction with solvents. The extraction of lycopene was achieved by treatment with ethanol and hexane and was repeated twice. Hexane phases were combined and the concentration of lycopene was determined in cells by spectrophotometry at 472nm. The viability of cells were analysed by tryphon blue assay[23]. The results were expressed as the percentage of the control culture treated with vehicle (Tween 40) alone.

MTT cell cytotoxicity assay

The 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was carried out by the method of Edmonson et al[24]. Cells cultivated in 96 well plates were washed with PBS and replenished with fresh culture medium. The six wells treated as control group contained cells with MTT alone. The second group of six wells cells was incubated with H_2O_2 and MTT. The third group of six well contained

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cells with lycopne and MTT. The cells of the fourth group of six wells were preincubated with 10µM of lycopne for twelve hours for maximum incorporation before the addition of the chemical oxidant such as H_2O_2 (500 microM). The second and fourth groups of wells were incubated with the subleathel dose of H_2O_2 for 24 hrs and the cells were washed with PBS and 20 µl of 5 mg/ml MTT was added to each well. The reagent blank well was with MTT but no cells. The contents were incubated for 3.5 hours at 37°C in culture hood and then media was removed carefully followed by the addition of 150 µl MTT solvent. The absorbance was read 590 nm with a reference filter of 620 nm. Background absorbance values consisted of reagent blank wells (with no cells) into which medium, MTT dye, and MTT solvent buffer were added. The background readings were subtracted from the average absorbance readings of the treated wells to obtain an adjusted absorbance reading that represented cell viability. This reading was divided by the adjusted absorbance reading of untreated cells in control wells to obtain the percentage of cell survival. Results were expressed as the percentage of surviving cells relative to the control samples.

Statistical analysis

The data analysis was done by using the statistical software SPSS 16 (Inc., Chicago, IL) for windows. Six samples of each 3different concentration (0 µm (control), 5 µm, 10 µm and 15µm) for 4 different time intervals (3hrs, 6 hrs, 12 hrs, and 24hrs) were analyzed for statistical significance. The descriptive statistics (mean and SD) were calculated for percent viability and percent incorporation during 4 different time periods for all the 3 concentrations of lycopene. The percent viability of different concentrations of lycopene during different time periods was analyzed by using two-way repeated measures ANOVA and post-hoc analysis was done with Bonferroni adjustment. One way ANOVA was used to analyze the percent viability for MTT cell cytotoxicity assay and post-hoc analysis was done by Dunnet post-hoc test.

RESULTS AND DISCUSSION

A number of studies have indicated the health benefits of consuming lycopene, in the diets of people in developed and developing countries[25]. Lycopene and a diverse range of carotenoids were identified and quantified in the human ciliary body and RPE/choroid[26]. The consumption of lycopene-rich foods has been demonstrated to prevent the occurrence of a number of chronic diseases[27,28]. Figure 1 exhibits the effect of lycopene concentration on the cell viability at different time intervals. The result clearly reveals that lycopene (5–15µM) treatment did not significantly affect cell viability in the trypan blue exclusion (cell counting) assays. The cell viability was neither increased nor decreased significantly

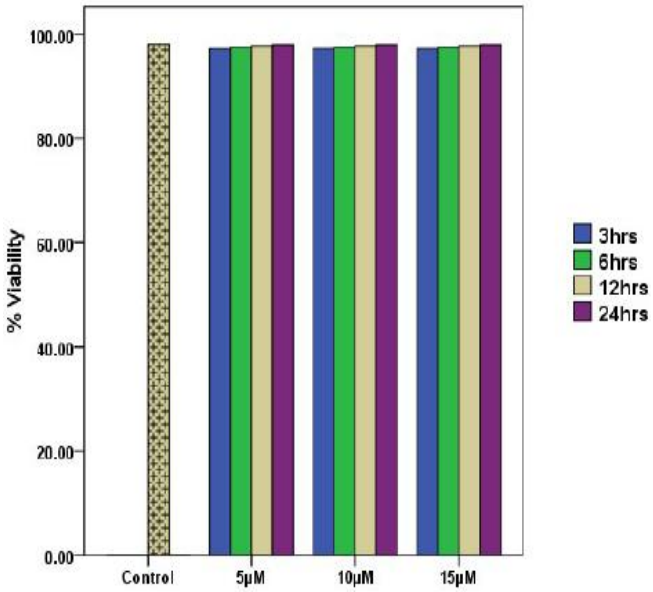

Figure 1 : Effect of different concentrations of Lycopene on cell viability

when compared to that of the control. This finding matches with the earlier findings of Chi-Ming Chanet al., and Richards LR et al[29,30] and clearly establishes the nontoxic nature of lycopene[31].

There was no statistically significant difference found between the groups at 0.05 level (P<0.0001).

Figure 2 indicates the pattern of lycopene incorporation to the cells. The data reveals that the lycopene uptake was dose and tim dependent. The lycopene incorporation values were significantly increased at a dose of 10µM lycopene than 5 µM, when compared with the control. 24.33% nd 53.50 of lyopene incorporation was achieved with 5µM and 10µM respectively. There was no further significant uptake of lycopene observed at ahigher

concentrations of 15 µM. This may be due to the fact that at higher concentration lycopene forms

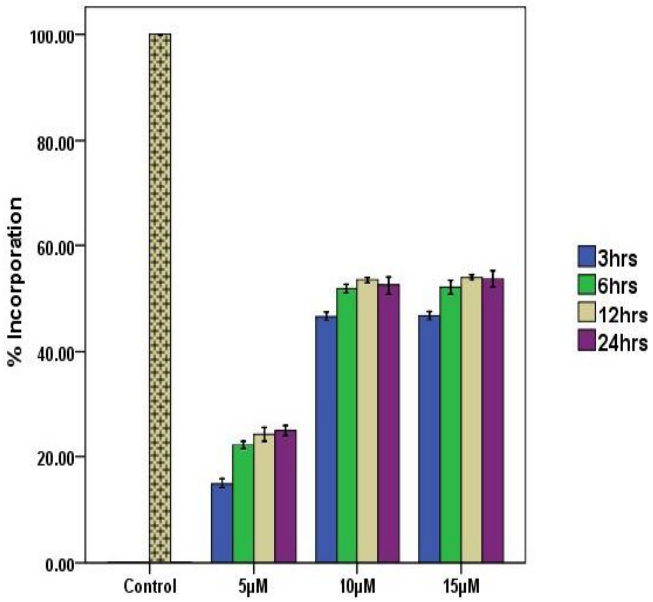

Figure 2 : Time and dose dependant incorporation of Lycopene on RPE cell line

more microcrystallines on the cell membranes that cannot be easily taken up by the cells. Similarly the maximum incorporation of lycopene was achieved at 12 hours of incubation after which there was no significant increase in the lycopene incorporation was observed.

The pair wise comparison made with Bonferroni adjustment for the groups revealed a statistically significant difference (P<0.0001) between all the pairs except between 10µM and 15µM concentrations of lycopene in all the time periods at 0.05 level. When comparing the unique pairs of different time periods with Bonferroni adjustment, all the pairs showed a statistically significant difference except the pair, 12hrs vs. 24hrs in all the 3 different concentrations at 0.05 level. The error bars displayed in the above figure represent ±2 SD.

The antioxidant property of lycopene was well established from the results of MTT cell cytotoxicity assay (Figure 3). Oxidative damage to the RPE is a potential final common pathway for age-related retinal damage that depends on free radical injury and many other factors[32,33]. The administration of H_2O_2 exerts oxidative stress on the RPE cells through the production of Reactive Oxygen Species. These species reacts, for example, with nucleic acids, membrane lipids, surface proteins, and integral glycoproteins of RPE cells and mediates the damage[34]. The oxidative damage to the RPE cells results in the loss of viability (55.17%) which was well documented in this study. However lycopene pretreated cells significantly increases the cell viability (77.42%). This function of lycopene is due to its antioxidant nature[35]. Lycopene is effective antioxidant and able quencher of singlet oxygen and other ROS[36,37]. The oxidative stress exhibited by the H_2O_2 was well scavenged by the lycopene and resulted in the maintenance of cell viability to the near normal value of control. The treatment of Lycopene alone to the cells does not influence the cell viability in a significant way. This finding coincides with the results of the tryphon blue assay and confirms non toxic nature of lycopene. One of the underlying hypotheses for

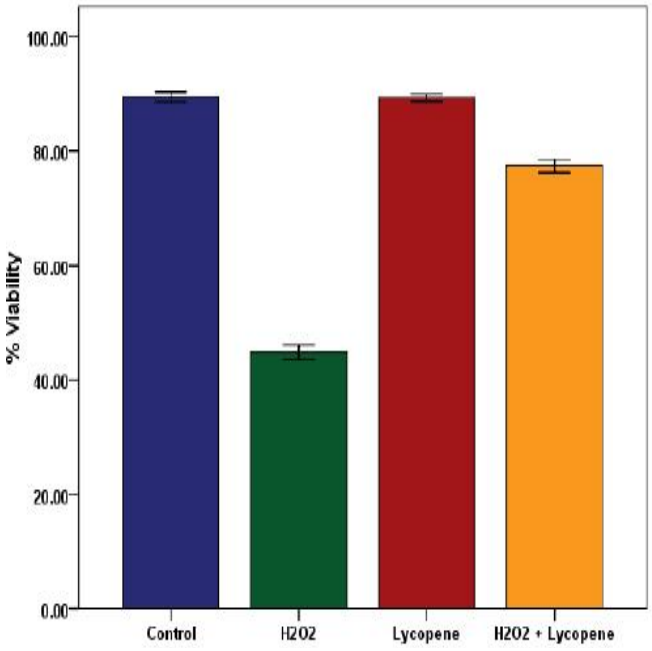

Figure 3 : Antioxidant activity of lycopene against H_2O_2induced oxidative stress on RPE cells.

the protective role of carotenoids in age-related Macular Degeneration (AMD) and cataracts has been based on the ability of these carotenoids to act as antioxidants that can protect the human retina from photo-oxidation. The results of the present investigation upholds this hypotheses by establishing the antioxidant effect of lycopene on

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RPE cell line and needs further insight to explore the preventive effect of lycopene against the progression of AMD.

When comparing the groups (H_2O_2, lycopene alone, and H_2O_2 plus lycopene) independently with control group by using Dunnet post-hoc test, except the group treated with lycopene, H_2O_2group ($P<0.0001$) and H_2O_2 plus lycopene group ($P<0.0001$) showed a statistically significant difference with control group at 0.05 level. The error bars displayed in the above figure represent ±2 SD.

CONCLUSION

Lycopene, a natural carotenoid pigment is long known for its potent antioxidant nature and provides significant health benefits against variety of diseases. This antioxidant is consumed through diat and gets widely distributed in different tissues and organs of the body including eye. In the present investigation it has been found that lycopene was incorporated to the RPE cell lines effectively and does not exhibit any kind of toxicity to the cells. The presence of lycopene in the RPE cell lines offer a significant protection against the oxidative damage exerted by H_2O_2. Thus lycopene is an able scavenger of free radical mediated stress and promising candidate to halt the progression of ocular damage in AMD.

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