ORIGINAL ARTICLE

EFFECT OF VITAMIN C AND E ACTIVITY ON SURGICALLY REMOVED CATARACTOUS HUMAN LENS EPITHELIUM CELLS

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ABSTRACT

Oxidative stress has been proposed as a common underlying mechanism of cataractogenesis. Experimental and observational data suggest that micronutrients like vitamin C and vitamin E with antioxidant capabilities may retard the development of age-related cataract. Effect of these factors on lens epithelium cells, center of lens metabolic activities, is not completely elucidated. The aim of present study was to examine the effect of vitamin C and E on surgically removed lens epithelium cells of patients with cataract. Capsulorhexis samples were collected from 170 patients, admitted for cataract surgery. Catalase specific activity was estimated in lens epithelium cells with and without vitamin (C or E) treatment at different concentration for different time duration. Student's t-test was employed for data analysis. We observed that in ex-vivo condition, a) both vitamin C and E bring about a decrease in catalase activity in lens epithelial cells. b) vitamin C showed toxic effect at high concentration. c) 100 μ M was the optimum concentration at which both vitamins showed maximum antioxidant activity. It was concluded that both vitamin C and E has direct effect on lens epithelium cells. At optimum concentration, they can reduce oxidative stress in these cells thus can support to prevent or delay cataract development.

KEY WORDS

Cataract, Lens epithelium, Vitamin C, Vitamin E.

INTRODUCTION

Cataract is an opacification of the lens that causes decreased visual acuity and can lead to blindness. It becomes more common with increasing age and is an important cause of disability among older adults; with increasing number of cataract surgeries being performed annually in the India. Recent findings suggest that the Indian population is either

widely exposed to the environmental risk factor and /or there is a genetic predisposition. In a recent study on the North Indian rural population, the prevalence of cataract in the age group >50 years was 75.3% (1) which appears to be highest in the world. Thus, identification of nutritional factors that could delay or prevent cataract development would be important both, for increasing the well-being of older adults and for reducing medical care costs.

Oxidative damage plays a major role in cataractogenesis, occurs when the level of pro-oxidants (reactive oxygen species and other free radicals) exceeds the ability of the cell to respond through antioxidant defense and ultimately leads to modification and degradation of protein, damage to DNA and mitochondria, and cell death (2). Much evidence suggests that elevated intakes or plasma concentrations of antioxidants are associated with decreased risk of cataract (3,4). Antioxidant vitamins (C and E) have been shown to prevent cataract

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formation by blocking the oxidative modification of lens protein and lipid within the epithelium of the lens in many experimental models (*in vitro*) (5) and animal models (*in vivo*) (6). A fall in these vitamins's level in ocular humors and lens were also observed in cataract patients (7).

The crystalline lens is an encapsulated and avascular area, composed mostly of protein and water, forms structurally clear tissue allowing light to pass through and focus on the retina. It contains a single layer of epithelial cells (LEC) that, in the equatorial region, terminally differentiate into fiber cells and are center of metabolic activity of lens therefore have great significance in stress-induced development of cataract. The LECs are the first site of oxidative damage, which then ultimately affects the entire lens and leads to pathogenesis of cataract (8). Previous studies have reported a protective effect of these vitamins in guinea pigs, rat and rabbit. However, in humans, the studies are confined to epidemiological data of nutritional intake of vitamins (9) and aqueous concentration of vitamins (10) in relation to cataract. There is no direct evidence showing the protective effect (of vit. C & E) on the stress induced human lens epithelial cells. Hence the present study was undertaken to see the effect of Vitamin C and E on human cataractous lens epithelial cells.

MATERIALS AND METHODS

Patient Selection: This prospective observational ex-vivo study comprised 170 consecutive patients with uncomplicated age-related cataract who had undergone phacoemulsification. Informed consent was obtained from all patients. Only patients older than 50 years with pupils dilating more than 7.0 mm and with otherwise normal eyes were included in the study. Exclusion criteria were diabetes mellitus, hypertension, glaucoma, shallow anterior chamber, uveitis, high myopia (axial length ≥ 27.0 mm), pseudoexfoliation, traumatic cataract, subluxated cataract, previous ocular surgery, ocular disease, steroid or immunosuppressive therapy, and allergy to dilating drops.

Collection and processing of Samples: Circular pieces of cataractous human anterior lens capsule (rhexis), about 6mm in diameter, were obtained post operatively in sterile normal saline. The cataract type was noted using the LOCs classification system (11). The anterior capsules taken for all the experiments belonged to pure senile mixed type (nuclear+cortical) of cataract. A single rhexis was placed in 1 ml Eagle's minimal essential medium (MEM) containing 10% fetal calf serum in a single well of 24 wells plate. The acclimatization was done by incubating the LECs for 30 min

in a 5% CO₂ incubator at 37°C.

Cell Viability Test / Typan blue exclusion test: **Typan blue exclusion test** used to insure cell viability. In this test, few drops of 0.025% typan blue in PBS is added on a rhexis, placed on glass slide, and then microscopically examined after 3 mins to determine whether cells taken up or excluded dye. Blue cells were then counted and these counts were used to calculate the relative proportion of the dead cells.

Treatment of human lens epithelial cells with vitamin: Following acclimatization the media was replaced by fresh media containing vit.C (100µM). After 1min. of exposure, rhexis was taken out with help of forcep and kept in 50µl PBS for 20 min. at -20°C. Similarly, other rhexis samples were exposed to different concentration of vit.C and E (at concentration 10, 100 & 1000µM) for 1, 5 and 10 minutes in individual wells separately (details described with results). After each treatment for the required time period, all samples were then processed individually for estimation of catalase activity. A single rhexis (total six separately) was used for each treatment.

Catalase assay: Catalase activity in LEC was assayed using Luck's method (12). The enzyme extract was prepared by homogenizing the LECs in 100ml lysis buffer [0.25 M sucrose, 20mM tris-HCl, 100mM KCl, 40mM NaCl and 10mM MgCl₂], centrifuging them at 20,000xg for 30 minutes at 4°C, and re-extracting the pellet in 100ml microsomal dilution buffer [0.1M KH₂PO₄, 20% glycerol, 10mM EDTA and 0.1mM β-mercaptoethanol]. 50µl of the pooled extract was added to 950µl of 10mM H₂O₂ and the rate of decomposition of H₂O₂ was measured spectrophotometrically at 240nm. Corresponding blanks containing 50µl of homogenizing buffer were run and blank readings were subtracted from the actual readings while calculation. The assay was performed three times from each homogenized enzyme extract and the remaining 50µl was used for protein estimation. The enzyme units /ml were calculated using the definition "decomposition of 1µM H₂O₂ in 1 minute corresponds to one unit of catalase" and specific activity was defined as units/mg protein. Catalase specific activity was calculated in LEC by measuring protein by eosin Y method (13).

RESULTS

Total 170 rhexis samples were collected from the patients having mean age 66.69 ± 8.37 (±SD) years for males and 62.47 ± 10.07 years for females. They (males and females) were approximate equal in number. Total 120 samples were used to study the effect of vitamins and other was used to

establish the cell viability, acclimatization and standardization of different procedures.

Cell viability and Acclimatization: The time required for the LECs to remain viable in PBS, was calculated to decide on the processing time of LECs. It was scrutinized that lens epithelial cells present in capsulorhexis remain viable upto 2 hour in PBS as shown in Figure 1A. More than 90 % cells have clear cytoplasm (live cells) up to 2 hour whereas remaining cells pick up the dye (dead cells). LECs rapidly died after 2 hour in PBS (Figure 1B). In next step, LEC were placed in MEM for different time period and catalase activity was estimated. No significant change in catalase activity was observed up to 1 hour and after that it started to decrease (data not shown). So we kept LEC in MEM for 30 minutes to remove artificial oxidative stress (induced by surgery) and termed this step as acclimatization of LEC.

Effect of Vitamin C and E on LEC: The effects of exposure of these vitamins at different concentration for different time periods on LECs (in term of catalase activity) are shown in Table 1. Each value shown is 'Mean $\pm$ SD' of six experiments. Significance of difference was measured by student's t-test.

Fig 1: Rhexis stained with typan blue. (A) Lens epithelial cells in PBS are viable upto 2 hour and (B) died rapidly after 2 hour. Plane arrow shows dead cells while arrow with dot shows live cell

Fresh sample, in which catalase activity was estimated immediately after acclimatization, was taken as atypical control (AC) and it was found 476.38 ± 38.71 U/ μ g of protein. Whereas in typical control (TC), samples were transferred in the MEM media and placed in CO₂ incubator for 48 hours following standard protocols, catalase activity was 97.27 ± 32.61 U/ μ g of protein.

Effect of Vitamin C: LEC samples were placed in complete media (one sample/ml/well) for 1 minute, which contained 10 μ M, 100 μ M and 1000 μ M vitamin C separately. The catalase activity in vitamin C treated samples (for 1 min) was found to be lower than AC but still higher than TC. For further evaluation another set of samples were placed in complete media with same concentration of vitamin C for different time periods (1, 5 and 10 minutes). With 10 μ M vitamin C, mean catalase activity remains approximately same with 1 min and 5 min treatment. It was decreased in 10 min but still very higher than TC. With 100 μ M vitamin C, catalase activity of LEC deceased continuously with time and after 10 mins it was reached at normal level. A reverse and adverse effect was observed at 1000 μ M vitamin C. The catalase activity, enormously increased with time, reflects a toxic effect.

Effect of Vitamin E: Samples were treated with vitamin E in same pattern as followed with vitamin C. There was a decrease in catalase activity with each concentration of vitamin E within 1 min. With 1000 μ M vitamin E, activity was found to be less than TC. For further evaluation samples were exposed for 1, 5 and 10 minute time period at each concentration. Vitamin E produced an antioxidant effect at concentration of 10 μ M as there was a decrease in catalase activity at each time period and activity came to normal level after 5 & 10 mins treatment. With 100 μ M, decrease in catalytic activity followed linear pattern with time. It was decreased significantly within 5 minute and reached up to the normal level with 10 minute treatment. 1000 μ M concentration seems an overdose of vitamin as the activity become normal within 1 min treatment and remains constant and significantly less than TC.

Comparison of effect of both vitamins at optimum concentration: Both vitamins showed antioxidant activity at optimum concentration (100 μ M for both). Vitamin C found to be effective in long term exposure (5 and 10 mins.) whereas vitamin E is efficient at both short (1 min) and long term exposure (Figure 2).

DISCUSSION

An inverse association with cataract and blood antioxidants

Fig 2: Comparison of effect of vitamin C and vitamin E activity at optimum concentration

in an antioxidant-depleted population is recently reported (14). *In vivo* studies of vitamin C in guinea pigs (6) and vitamin E in rat (15) have demonstrated a protective role of these compounds. *In vitro* studies yielded similar results (16). The question remains however whether comparable effects occur in senile cataracts in humans. The present study is the first of its kind showing the antioxidant role of these vitamins directly on stressed (cataractous) human LECs. LECs are the first site of oxidative damage and any injury to LECs results in lens opacification (8). Hence, the present study was performed using LECs obtained from surgically removed cataractous epithelium. The use of similar samples for documentation of various enzymes has been done by the same group (17).

In previous studies, either lens cells (11) or cultured lens

epithelial cells (2) were employed to examine the effect of these free radical scavenger vitamins or other antioxidant substances. In lens, only lens epithelial cells are metabolically active. Other lens cells do not contain cell organelles and cultured lens cells may not produce the same results (like *in-vivo* cells) as they have developed in most favorable environment. Moreover these cells were treated with H_2O_2 to produce artificial oxidative stress for these *in-vitro* studies (2). In present ex-vivo study it was attempted to maintain the same cellular environment (that exists *in vivo*) by acclimatization and by measuring LEC enzyme without giving any artificial stress. It was first assured that these LECs have high oxidative stress as they come from cataractous patients and this is evident from the high catalase activity in these samples (AC). So no artificial oxidative stress inducer was used. For normal or typical control (TC), some other samples are kept in MEM culture medium for 48 hr following standard protocols. The presences of optimum condition, suitable environment and restoration of micronutrients remove oxidative stress results in the lower levels of catalase activity compared to the fresh samples (Table-1). The same model has been used previously by our co-authors to show the protective effect of estrogen (18). Two major antioxidative enzymes used by the lens are catalase and glutathione peroxidase (19). The role of catalase in defending cells and tissues against oxidative stress has been studied extensively. As expected, overexpression of catalase renders cells more resistant to toxicity of H_2O_2 and oxidant mediated injury from exposure to hyperoxia (20). It is also known that glutathione peroxidase plays a major role in removing H_2O_2 present in low concentrations in cells whereas catalase is more effective with high concentrations of H_2O_2

Table 1: Catalase activity observed after treatment of LECs with different concentrations of vitamin for 1 minute, 5 minutes and 10 minutes

	1 minute	Catalase activity (U/ μ g of Protein) 5 minute	10 minutes
Atypical control (AC)	476.38 $\pm$ 38.71		
Treatment with different concentration of vitamin C (μ mole)			
10	283.23 $\pm$ 36.99*	312.21 $\pm$ 39.82 ^{NS}	228.88 $\pm$ 29.76 ^{##}
100	403.81 $\pm$ 30.57**	100.63 $\pm$ 22.51 [#]	57.8 $\pm$ 11.50 [#]
1000	310.62 $\pm$ 23.87*	878.31 $\pm$ 109.38 [#]	1800.61 $\pm$ 340.64 [#]
Treatment with different concentration vitamin E (μ mole)			
10	158.14 $\pm$ 20.44*	44.19 $\pm$ 22.78 [#]	48.17 $\pm$ 13.23 [#]
100	145.92 $\pm$ 22.82*	106.27 $\pm$ 15.70 ^{##}	63.58 $\pm$ 15.81 [#]
1000	59.78 $\pm$ 18.35*	54.18 $\pm$ 12.57 ^{NS}	45.06 $\pm$ 10.84 ^{NS}
Typical control (TC)	97.27 $\pm$ 32.61*		

Values are Mean $\pm$ SD. * P < 0.001, ** P < 0.01 significance of difference from AC; # P < 0.001, ## P < 0.05 significance of difference from catalase activity with 1 min treatment. NS : not significant.

(21). There is a significant increase in the H_2O_2 concentration during cataractogenesis (7), hence catalase was preferred to glutathione peroxidase and used as an indirect marker to evaluate the oxidative status of the sample.

In LECs, both vitamins are present in micro mole concentration (22). Previous study using the rabbit LECs also reported that pretreatment of cells with vitamin C (25-50 μM) or vitamin E (5-40 μM), restored the resistance of GSH-depleted cells to H_2O_2 (5) so 10, 100, and 1000 μM vitamin concentrations were selected for initial experiments in the present study. Short term (1 min.) exposure with both vitamin C and E at these concentrations reduces the catalase activity within LECs compare to AC and hence the antioxidant effect on LEC of these vitamins is very obvious. The optimum time to be effective was 1minute for vitamin E and 10 min for vitamin C. The probable reason for this time difference could be that vitamin E is fat-soluble and vitamin C is water soluble free radical scavenger (5) and hence vitamin E can easily penetrate the lipid bilayer of the cell membrane. However, the optimum concentration for both the vitamins appears to be same i.e. 100 μM in our results. Protective concentration from previous reports include 2.5mM of vitamin C for rat lens (23) 25-50 μM vitamin C and 5-40 μM Vitamin E in cultured rabbit lens epithelial cells (5). The effective concentration for both the vitamins appears to be higher than the already published reports. The probable reason for this could be that the cells used in the present study were from cataractous patients and surgically removed so as the level of oxidative stress is high compared to the normal cells, the vitamins concentration required to combat the stress is high. However, to the best of our knowledge we did not come across any reports showing the effect of these vitamins on human LECs. At 1000 μM concentration, vitamin C shows the deleterious effects as catalase activity was increased by many folds. The adverse effects of higher concentration of vitamin C are similar to previous findings in which $>400\mu M$ of vitamin C enhanced the toxic effect of H_2O_2 (5).

At high dose (1000 μM), vitamin E produced a drastic decrease in activity therefore considered as over dose. Furthermore it is not similar to previous findings that vitamin E concentration more than 80 μM leads to the toxic effect of H_2O_2 (5). In those studies either artificial oxidative stress was generated or antioxidant enzyme was depleted in cultured LEC by H_2O_2 treatment. This might change the response of LEC against vitamins E and C. Otherwise these discrepancies in the results of these studies are difficult to explain except on the grounds that the cells probably came from different origins and were in different conditions.

In the lens of experimental model a slight stimulation of antioxidant system by a small number of free radicals has been observed, which provokes a reaction of sweeping them away (24). Perhaps by the same mechanism catalase activity was increased to counteract the increased oxidative stress (in terms of H_2O_2). These results are in agreement with other groups worked on the same hypothesis and reported correlation of different enzymes and lipid peroxidation (25).

both vitamin C and vitamin E has direct antioxidant effect on surgically removed LECs thus can reduce the level of oxidative stress in vivo condition and may produce beneficial effects which can prevent or delay cataract development.

Catalase plays predominant role in removal of hydrogen peroxide in RBCs which are terminally differentiated cells like lens cells. In this context that H_2O_2 plays important role in cataractogenesis, how much a load lens catalase bears among other antioxidant enzymes in prevention of cataract? is still not answerable. So it will be interesting to find out comparative significance of different antioxidant enzymes against development of cataract.

REFERENCES

1. Murthy GV, Gupta SK, Maraini G, Camparini M, Price GM, Dherani M, et al. Prevalence of lens opacities in North India: the INDEYE feasibility study. *Invest Ophthalmol Vis Sci* 2007; 48 (1): 88-95.
2. Doshna CM, Shen AC, Gebhard DF, Brees DJ, Soms CJ. Investigating Markers of DNA Oxidation, Lipid Peroxidation and Stress Response in Human Lens Epithelial Cell Lines. *Invest Ophthalmol Vis Sci* 2005; 46: E-Abstract 3850.
3. Jacques PF, Chylack LT. Epidemiologic evidence of a role for the antioxidant vitamins and carotenoids in cataract prevention. *Am J Clin Nutr* 1991; 53: 352S-5S.
4. Mares-Perlman JA, Brady WE, Klein BEK, Klein R, Haus GJ, Palta M, et al. Diet and nuclear lens opacities. *Am J Epidemiol* 1995; 141: 322-34.
5. Shang F, Lu M, Dudek E, Reddan J, Taylor A. Vitamin C and E restore the resistance of GSH- depleted lens cells to H_2O_2 . *Free Radical BioMed* 2003; 34 (5): 521-30.
6. Blondin J, Baragi VK, Schwartz ER, Sadowski J, Taylor A. Prevention of eye lens protein damage by dietary vitamin C. *Invest Ophthalmol Vis Sci* 1986; 27: 9-14.
7. Bhuyan KC, Bhuyan DK. Molecular mechanism of cataractogenesis-III. Toxic metabolites of oxygen as initiators of lipid peroxidation and cataract. *Curr Eye Res* 1984; 3(1): 67-81.

8. Spector A, Wang GM, Wang RR, Li WC, Kuszak JR. A brief photochemically induced oxidative insult causes irreversible lens damage and cataract. I. Transparency and epithelial cell layer. *Exp Eye Res* 1995; 60: 471-81.
9. Bates CJ, Chen SJ, Macdonald A, Holden R. Quantitation of vitamin E and a carotenoid pigment in cataract's human lenses, and the effect of dietary supplement. *Int J Vitam Nut Res* 1996; 66: 316-21.
10. Tessier F, Moreaux V, Birlouez-Aragon I, Junes P, Mondon H. Decrease in vitamin C concentration in human lenses during cataract progression. *Int J Vitam Nutr Res* 1998; 68(5): 309-15.
11. Chylack LT Jr, Wolfe JK, Singer DM, Leske MC, Bullimore MA, Bailey IL, et al. The Lens Opacities Classification System III. The Longitudinal Study of Cataract Study Group. *Arch Ophthalmol* 1993; 111(6): 831-6.
12. Luck H. Catalase. In: Bergmeyer HU, editor. *Methods of enzyme analysis*. Academic Press: New York; 1971: 885 pp.
13. Waheed AA, Gupta PD. Estimation of submicrogram quantities of protein using the dye eosin Y. *J Biochem Biophys methods* 2000; 42:125-32.
14. Dherani MK, Murthy GV, Gupta SK, Young I, Maraini G, Camparini M, et al. Blood levels of vitamin C, carotenoids and retinol are inversely associated with cataract in a north Indian population. *Invest Ophthalmol Vis Sci* 2008; 17:1202.
15. Mathew JP, Thomas VC, Thomas I. Selenite cataract and its attenuation by vitamin E in Wistar rats. *Ind J Ophthalmol*. 2003; 51(2): 161-70.
16. Stewart-DeHaan PJ, Creighton MO, Sanwal M, Ross WM, Trevithick JR. Effects of vitamin E on cortical cataractogenesis induced by elevated temperature in intact rat lenses in medium 199. *Exp Eye Res* 1981; 32 (1): 51-60.
17. Alapure BV, Praveen MR, Gajjar D, Vasavada AR, Rajkumar S, Johar K. Matrix metalloproteinase-9 activity in human lens epithelial cells of cortical, posterior subcapsular, and nuclear cataracts. *J Cataract Refract Surg* 2008; 34(12): 2063-7.
18. Gajjar D, Patel D, Alapure B, Praveen MR, Patel A, Johar K, et al. Rapid action of oestradiol against hydrogen peroxide-induced oxidative stress in cataractous lens epithelium: an in vitro study. *Eye* 2008; 23: 1456-63.
19. Spector A, Ma W, Wang RR, Yang Y, Ho YS. The contribution of GSH peroxidase-1, catalase and GSH to the degradation of H₂O₂ by the mouse lens. *Exp Eye Res* 1997; 64: 477-85.
20. Lindau-Shepard BA, Shaffer JB. Expression of human catalase in acatalasemic murine SV-B₂ cells confers protection from oxidative damage. *Free Radic Biol Med* 1993; 15: 581-8.
21. Costarides AP, Riley MV, Green K. Roles of catalase and the glutathione redox cycle in the regulation of anterior-chamber hydrogen peroxide. *Ophthalmic Res* 1991; 23(5): 284-94.
22. Krepler K, Schmid R. Alpha-tocopherol in plasma, red blood cells and lenses with and without cataract. *Am J Ophthalmol*. 2005; 139(2): 266-70.
23. Varma SD, Kumar S, Richards RD. Light-induced damage to ocular lens cation pump: prevention by vitamin C. *Proc Natl Acad Sci USA* 1979; 76(7): 3504-6.
24. Sztarbala T, Gos R, Kedziora J, Blaszczyk J, Sibinska E, Goralczyk M. Changes in the antioxidant system of the aqueous humor, lens and erythrocytes after sulfur hexafluoride. *Klin Oczna* 1998; 100: 73-5.
25. Klos-Rola J, Zagorski Z. Peroxidation of lipids in patients with senile cataract. *Klin Oczna* 2004; 106: 416-8.