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ORIGINAL ARTICLE

A Potential Correlation between Systemic Oxidative Stress and Intracellular Ambiance of the Lens Epithelia in Patients with Cataract

GOYAL M M *, VISHWAJEET P*, MITTAL R** , SUNE P***

ABSTRACT

Aim: To study the correlation between systemic oxidative stress and intracellular ambiance of the lens epithelia in patients with cataract.
Materials and Methods: Spectrophotometery was employed for the estimation of catalase activity and the extent of lipid peroxidation in the lens epithelial cells (LEC) and plasma. Both are markers for oxidative stress. No antioxidant medicines were used by the cataract patients enrolled in this study; otherwise, they were all healthy individuals without any systemic diseases.
Results: A total of 56 patients with cataract were included in this study. The mean ages of the patients were 66.6 ± 8.3 (±SD) years for males and 62.4 ± 10.0 years for females. Catalase activity was estimated in surgically removed LEC (221.16±135.87 U/µg protein; Mean±SD) and in plasma (277.56±162.44 KU/mg Hb). MDA levels were also calculated in LEC (1.28±0.79nM/mg protein) and in plasma (537.30±238.47 nM/g protein). Linear regression analysis showed a partial positive correlation in LEC and plasma catalase activity (r: 0.701; $p<0.05$), but not in MDA (r: 0.248; $p>0.05$).
Conclusion: Increased systemic oxidative stress can lead to the development or progression of cataract by affecting the intracellular ambiance of the lens epithelia. So, subjects having high systemic oxidative stress are more vulnerable for the development of cataract.

Key Words: cataract formation, lens epithelium, catalase, lipid peroxidation.

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Introduction

Cataract is the leading cause of blindness worldwide. In India, contemporary findings suggest that the population is either widely exposed to the environmental risk factors 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 the highest in the world. Even with all resources available, clinicians are finding it hard to eradicate cataract induced blindness completely [2]. Therefore, it is indispensable to find out the causative factors for the prevention of the development of cataract.

Several risk factors have been identified for the development of human cataract: aging, diabetes, malnutrition, poverty, sunlight, smoking, hypertension, and renal failure [3]. Although cataract is a multifactorial disease, oxidative mechanisms are believed to play an important role in the pathogenesis of maturity-onset cataract, the most important cause of visual impairment at an advanced age**.** Oxidative stress (OS) occurs when the level of pro-oxidants (reactive oxygen species and other free radicals) exceeds the ability of the cell to respond through antioxidant defenses and ultimately leads to the modification and degradation of protein, damage to DNA and mitochondria and cell death [4]. The increased production of free radicals and the oxidation of unsaturated lipids have been observed in cataractous lenses [5] and aqueous humour. Lens epithelial cells (LEC) are the main site of oxidative damage, which then ultimately affects the entire lens, thus affecting the lens clarity and leading to the pathogenesis of cataract [6], [7].

On the other hand, since many years, experimental evidences have suggested an association between nutrition and lens opacities. Increased systemic OS has been observed in cataract patients (8]. In some other studies, the parameters of their antioxidative defenses are lower and those of OS are higher in serum, in the lens and in the humour aqueous [9].

But systemic stress (oxidative) and its effects on the intracellular ambiance of the lens epithelia have not been correlated directly in pathological human samples. Therefore, the present study was undertaken to examine the correlation between oxidative stress in surgically removed LEC and plasma of patients with cataract.

Materials and Methods

Patient Selection

This study comprised of 56 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. The exclusion criteria were diabetes mellitus, hypertension, glaucoma, shallow anterior chamber, high myopia (axial length O27.0 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 [10]. The anterior capsules taken for all the experiments belonged to immature senile cataract. For acclimatization, a single rhexis was placed in 1 ml of Eagle's Minimal Essential Medium (MEM) containing 10% foetal calf serum in a single well of a 24 well plate and was incubated for 30 min in a 5% CO_2 incubator at 37^0C.

Fasting blood samples were collected in a glass bulb containing EDTA from patients, and their verbal consent was taken. The plasma was separated and stored at -20 °C until analysis.

Cell Viability Test

The Typan blue exclusion test **was** used to ensure cell viability. In this test, a few drops of typan blue is added on a rhexis, placed on a glass slide and then microscopically examined to determine whether cells take up or exclude the dye. A viable cell will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm.

Biochemical Assay

The level of malondialdehyde (MDA) was measured as an index of peroxidation of lipids by using the method of Beuge and Aust [11]. The catalase activities of plasma

and LECs were measured by Luck's method [12]. The catalase specific activity was calculated in LEC by measuring protein using the Eosin Y method [13] and in plasma by estimating haemoglobin using the O-tolidine method [14]. The methods are described here in brief.

Catalase Assay

The enzyme extract was prepared by homogenizing the cells in a lysis buffer (0.25M sucrose, 20mM tris-HCl,100mM KCl, 40mM NaCl, and 10mM $MgCl_2$), centrifuging them at 20 000 g for 30 min at 41C and by re-extracting the pellet in a microsomal dilution buffer (0.1M KH2PO4, 20% glycerol, 10mM EDTA, and 0.1mM b-mercaptoethanol). The pooled extract was added to 10mM of H_2O_2 and the rate of decomposition of H_2O_2 was measured spectophotometrically at 240 nm. The decomposition of 1 mM of H_2O_2 in 1 min corresponds to 1U of catalase.

Lipid peroxidation / MDA assay

TBA-TCA- HCl reagent (0.8 ml) was added to 0.2 ml of plasma. The mixture was boiled in a boiling water-bath for 15 min. After centrifugation, the absorbance of the supernatant was recorded spectrophotometrically at 535 nm. A blank (saline) absorbance due to reagents was subtracted from the corresponding experimental sample. The MDA content in the sample was calculated using an extinction coefficient of 1.56×10^5 /M/cm. at 535 nm. In LEC, the cells were homogenized in distilled water and were centrifuged at 12,000 g at 4°C for 30 min. Aliquots of this homogenate were used as the sample instead of plasma in the above procedure.

Estimation of Protein

100µl of the sample was added to 1.0 ml of the reagent (0.012% Eosin Y Dye and 0.6% Citric Acid; pH: 2.6-2.8) and was incubated for 15min at room temperature. The absorbance was measured spectrophotometrically at 543nm.

Estimation of Plasma Haemoglobin

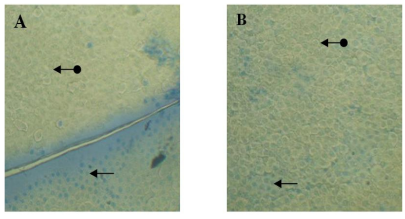
1.0 ml of working solution [0.4 gm % o-tolidine, 0.1% triton x-100 in solution (20:80) of glacial acetic acid and ethanol] and 1 ml of freshly prepared H_2O_2 solution [2% H_2O_2 and 2.26 % w/v sodium acetate in distilled water] were mixed in test tubes and this was kept for 5 min for maturation of the reagent. 10 µL of haemoglobin standards (6 to 400 mg/L) and samples were added into respective test tubes and immediately, ΔAbs per minute were measured at 630 nm with a delay time of 30 secs.Distilled water was used as the reagent blank.

Results

A total of 56 rhexis samples were collected from patients with immature senile cataract, having mean ages of 66.6 ± 8.3 (±SD) years for males and 62.4 ± 10.0 years for females. They (males and females) were approximately equal in number. We analyzed 25 samples for MDA and 31 samples for catalase activity separately, in LEC and plasma of the same patients.

Cell Viability and Acclimatization

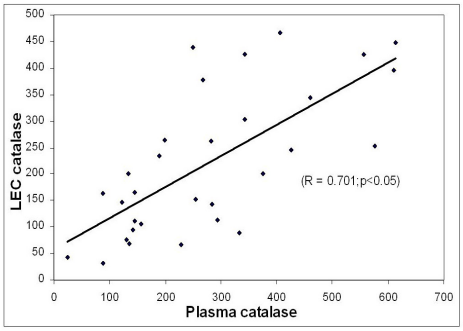
It was observed that the lens epithelial cells which were present in the capsulorhexis remained viable upto 2 hours in PBS, as shown in figure 1A. More than 90 % of the cells had clear cytoplasm (live cells) up to 2 hours, whereas the remaining cells picked up the dye (dead cells). LECs rapidly died after 2 hours in PBS (Figure 1B) [Table/Fig 1]. However, in the present study, it took a maximum of twenty minutes to enter the sample into the process after its removal from the eye.



(Table/Fig 1) Photograph of capsulorhexis 'A' upto 2 hour and 'B' after 2 hour in PBS. Plane arrow shows dead cells while arrow with dot shows live cell.

Correlation between Catalase Activity in Plasma and in LEC

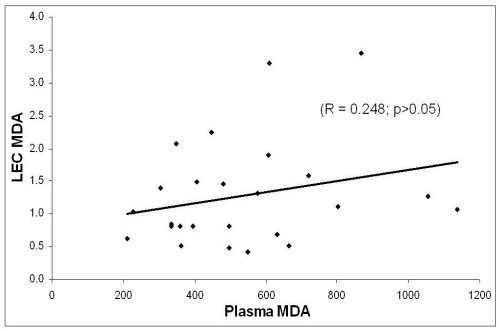
Catalase specific activity was measured both in LEC (mean ± SD; 221.16 ± 135.87 U/µg of protein) as well as in plasma (277.56 ± 162.44 KU/mg of Hb) of the same patient. A partial positive linear correlation was found (r: 0.701, $p<0.05$) between the catalase activity in LECs and in the plasma of the patients with cataract [Table/Fig 2].



(Table/Fig 2) Correlation of catalase specific activity in plasma and in LEC.

Correlation between MDA in Plasma and in LEC

MDA level was measured in LECs (1.282 ± 0.798 nM/mg protein; mean± SD) and in plasma (537.30 ± 238.47 nM/g protein) of the same patient. As shown in [Table/Fig 3], MDA levels in LECs are poorly correlated (positive) with plasma MDA levels. The correlation coefficient (r) was only 0.248. It was in accord with the results of other workers [15].



(Table/Fig 3) Correlation of MDA in plasma and in LEC.

Discussion

The elevated level of oxidative stress markers are reported in serum and RBCs in cataract patients. Experimental and observational data suggest that oral supplementation or increased blood level of micronutrients which can reduce oxidative stress, can retard the development of age-related cataract [16],[17],[18],[19].

In the present study, we attempted to find out the correlation between systemic and LEC oxidative stress. In the previous studies whole lens cells [5],[8] or aqueous humour samples were used to see the correlation of different factors in ocular tissue and blood. The generation of free radicals is an unwanted surplus of metabolism and it is a continuous process in living cells. Most of the lens cells (except epithelial cells) and aqueous humour do not contain cell organelles and so they are not metabolically active. Lens contents are a result of diffusion from LECs. So, appraisal of the effects of systemic oxidative stress on the lens leave a question as to how these cells which are not capable of general metabolism, react against stress.

Again, there are other factors also which can induce free radical generation in the surroundings of the lens, like UV light. The aqueous humour normally contains hydrogen peroxide (H_2O_2), a compound which is capable of generating reactive oxygen species (ROS). The systems protecting the ocular lens from these ROS (or oxidative damage) are primarily confined to the epithelium, a single layer of cells on the anterior side of the organ which directly lies beneath the lens capsule [20]. These LECs are centres of lens metabolic activities as they contain maximum mitochondria than any other parts of the lens [21]. Some data suggest that these cells are the initial site of attack by oxidative stress and follows the lens fibers, leading to cortical cataract [22],[23]. Hence, LECs have great significance in cataractogenesis and were employed to study the changes which were brought about by oxidative stress [22]. Cataract surgery can induce some stress in LEC [24]. To avoid this, the acclimatization step was introduced. The

same model was previously used by our co authors also [25],[26][,27].

Since ROS are highly unstable, their measurement in human serum or plasma samples does not necessarily reflect their in vivo concentration. Therefore, only secondary (end) products of oxidative stress have emerged for application in clinical studies. Malondialdehyde (MDA) is a naturally occurring product of lipid peroxidation, a well-established mechanism of cellular injury by ROS in both plants and animals and is used as an indicator of oxidative stress in cells and tissues.

H_2O_2 is the major oxidant involved in cataract formation [28]. There is a significant increase in the H_2O_2 concentration during cataractogenesis [17]. In in-vitro conditions also, relatively high concentrations of H_2O_2 are needed to cause significant changes in the lens epithelial cells [4]. Two major antioxidative enzymes used by the lens to combat H_2O_2 are catalase and glutathione peroxidase [29]. Glutathione peroxidase plays a major role in removing H_2O_2 which is present in low concentrations in cells, whereas catalase is more effective with high concentrations of H_2O_2 [30]. In another report, the cause of cell death following the inhibition of catalase was identified to be related to an inability of the cultured LEC to remove peroxide from the culture medium at a rapid rate, following the H_2O_2-pulse [31]. Catalase also protects other antioxidant enzymes from the destructive effects of H_2O_2 [32]. Hence, catalase was preferred to glutathione peroxidase and was used as an indirect marker to evaluate the oxidative status of the sample. In the lens of an experimental model, a slight stimulation of the antioxidant systems by a small number of free radicals was observed, which provoked a reaction of sweeping them away [33]. Perhaps by the same mechanism, its (catalase) activity is increased to counteract increased oxidative stress (in term of H_2O_2) either in LEC or in blood (systemic) [34].

Both catalase activity and MDA levels are good markers of oxidative stress. An increase in plasma catalase activity and MDA reflect increased systemic oxidative stress which might be the result of poor nutrition or smoking and tobacco chewing habits or environmental exposure, or all of them. Body tissue including LEC can react against oxidative stress in two ways. One is, by increasing the synthesis of antioxidant enzymes like catalase and glutathione peroxidase and the second is through more consumption of antioxidant substrates like reduced glutathione, vitamin C and vitamin E. Due to increased OS, the consumption of antioxidant substrates by body tissues increases and therefore, its availability is reduced across the blood brain barrier (BBB), a membranic structure that acts primarily to protect the brain from chemicals in the blood, while still allowing essential metabolic function. In this way, slight decrease in the availability of antioxidant substrates to LEC, which is continuously exposed to factors which provoke free radical generation like light, leads these cells to OS. If this condition persists for a long time, it affects the whole lens and its clarity by modification or degeneration of DNA, proteins and other biomolecules like lipids. These cells also respond by increasing the synthesis of catalase and if fails to respond, cataract develops. This is indicated by some findings where the catalase activity in cataract patients was significantly lower than in the control subjects [35]. Linear regression analysis results (Figure 2) endorsed the above said matter and found a correlation between the catalase activity in LEC and in the plasma of same patient ($r : 0.701$, $p<0.05$). These results are in agreement with those of other groups who worked on the same hypothesis and reported a correlation between different enzymes and lipidperoxidation products [36]. Some contradictory reports which are also available [6],[15], might be the result of the presence of factors other than elevated levels of H_2O_2. We could not find any report in which the catalase activities in surgically

removed LEC and in plasma were correlated.

Plasma MDA levels are poorly correlated (Figure 3) with MDA levels in LECs. In the previous studies, an inverse correlation of MDA and catalase activity has been reported. It is quite obvious as both are produced by opposite mechanisms. Lipid modification (peroxidation) by ROS results in an increase in MDA levels whereas increased catalase activity shows an elevated defense mechanism in the cells. This might be the reason for the poor correlation (Figure 3), as increased catalase activity decreases oxidative stress in LEC and inhibits lipid peroxidation (formation of MDA).

Although our results are from a relatively small sample size, they suggest some intriguing and potentially provocative findings which suggest that systemic oxidative stress can increase the same (OS) in LECs. This change in the intracellular ambiance of the lens epithelia can lead to modification of DNA, proteins and other biomolecules and can result in the development or progression of lens opacity. From this, we can conclude that individuals having increased systemic oxidative stress are more vulnerable to the development of cataract and so the oral supplementation or increased blood levels of the antioxidants may be beneficial in the prevention of cataractogenesis. Moreover, further studies with an increased sample size and different stress markers are needed to define the unambiguous role of oxidative stress in the development of cataract.

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