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COMPARITIVE STUDY OF OXIDATIVE STRESS MARKERS IN DIABETIC AND NON DIABETIC PATIENTS WITH CATARACT

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ABSTRACT

Purpose: This study compared the relationship of Malondialdehyde(MDA-a marker of oxidative stress) and Glutathione (GSH-which is a natural antioxidant) levels in blood and lens between diabetic patients with cataract and non-diabetic patients with cataract. **Methods:** In a prospective comparative study Blood and lens samples from thirty patients with diabetes and thirty patients without diabetes undergoing cataract surgery were collected and the levels of MDA and GSH were measured and compared and correlated. **Results:** There was significant increase in MDA levels in lens and blood in diabetic cataract patients. Levels of GSH in lens and blood were significantly lower in diabetic cataract patients.(p value <0.001) Positive 'r values' were obtained in the correlation between the levels of MDA in blood and lens as well as the levels of GSH in blood and lens. In contrast, the correlation between the levels of MDA and GSH in blood and lens separately gave negative 'r values' indicating a negative correlation between the two. **Conclusions:** The increased oxidative stress in the lens of diabetics may be primarily responsible for cataract formation in them at a younger age compared to non diabetics.

KEYWORDS: diabetes, cataract, oxidative stress.

INTRODUCTION

Cataract is a major cause of blindness worldwide and particularly so in India, it constitutes about 55% of total blindness.[1] The most recent estimates from WHO reveal that 47.8% of global blindness is due to cataract and in South Asia region which includes India, 51% of blindness is due to cataract.[2] In India alone, 4 million people turn blind due to cataract every year.[4]

It is generally acknowledged that age-related cataract is a multifactorial disease.[4] However, owing to inadequacies in epidemiologic understanding of this disease, many risk factors have been hypothesized, diabetes being the forerunner apart from the age itself. Cataract is also frequent in people in pre-diabetic state with abnormal glucose tolerance.[5]

The number of people with diabetes is increasing rapidly and there are an estimated 195 million diabetic patients worldwide which is also expected to rise to 366 million in 2030. India tops the list among all countries with an estimated 31.7 million diabetics and this number is expected to rise to 79.4 million by 2030.[6]

Cataract is considered a major cause of visual impairment in diabetic patients[6]. Studies indicate a three to fourfold increased prevalence of cataract in patients with diabetes under the age of 65, and up to a twofold excess above 65 years.[7]

Age related cataract is the most frequently seen variety and occurring at an earlier age in diabetic compared to non-diabetic patients. Cortical, nuclear and posterior subcapsular morphological varieties are more frequently seen. A special "snowflake cataract is mainly seen in young non-insulin dependent Diabetic patients.

Pathogenesis of cataract formation is a complex one and has not been understood completely, till date. Oxidative stress is one of the factors implicated in this disease. Oxidative stress may result when the cellular antioxidant defense mechanisms are unable to detoxify the free radicals and reactive oxygen intermediates. Peroxidation of lens fiber plasma lipids or lens fiber plasma membrane lipids has been suggested as a factor contributing to the crystalline lens opacification. In the process of lipid peroxidation, Malondialdehyde (MDA), a potent cross-linking agent is formed. It has been hypothesized that MDA cross-reacts with membrane lipids and proteins, rendering them incapable of performing their normal functions.

It is significant to note that oxidative stress is also implicated as the causative factor in many other diseases in the human body like cancer, atherosclerosis, Alzheimer's disease etc.[8]

The lens can protect itself against oxidation effectively by using various protective agents and enzyme systems which are the natural antioxidant systems. Of the several small molecular antioxidants, Glutathione (GSH) is perhaps the most important in the lens, along with ascorbic acid and Vitamin E.

Oxidative stress results in imbalance between radical oxidative species and cellular antioxidant mechanisms which leads to cellular damage, apoptosis and eventually cataract formation.[9]

AIM: To compare and correlate the relationship of Malondialdehyde and Glutathione levels in blood and lens between diabetic patients with cataract and non-diabetic patients with cataract.

MATERIAL AND METHOD

The study protocol was approved by the Ethics Committee of the institution. Written informed consent was taken from all the patients before enrolling them for the study. The enrolled patients were examined in the Ophthalmic Out-Patient Department (OPD) of the institution and were admitted for cataract surgery.

Group 1 – Thirty patients with diabetes and cataract.
Group 2 – Thirty non – diabetic patients with cataract.

Inclusion Criteria

1. Thirty cataract patients with diabetes mellitus of more than 5 years duration in Group 1.
2. Thirty cataract patients without diabetes mellitus in Group 2.
3. All the patients included in the study were above 50 years of age.

Exclusion Criteria

1. Patients with diabetes mellitus for less than 5 years duration.
2. Age less than 50 years.
3. Patients with history of ocular trauma, prior ocular surgery, glaucoma, uveitis, any systemic illness and on corticosteroid use.
4. Smokers, alcoholics and tobacco chewers.
5. Patients not willing for written informed consent.

All the enrolled patients were examined by an ophthalmologist and all the particulars of the patients were recorded in a pre-set proforma.

Sample Collection

A 2ml venous blood sample was collected in EDTA vacutainer from all the enrolled patients before they underwent cataract surgery. Subsequently all the patients underwent manual

small incision cataract surgery by a single ophthalmologist. The nucleus of the lens was collected after the surgery and placed in a tube containing Phosphate buffer (0.1 M, pH 7.4) which was immediately placed in an Ice box. The blood and nucleus were assayed for Glutathione (GSH) and Malondialdehyde (MDA) soon after collection on the same day.

Processing of Samples

Blood: The blood collected was centrifuged to separate the cells and plasma. The packed red blood cells were washed thrice in 0.9% saline. Then the cells were lysed with distilled water (1 volume cells+ 9 volumes of water), vortex mixed, centrifuged and the supernatant (hemolysate) was used for the assays of MDA and GSH.

Lens: The nucleus of the lens was homogenized 1:10 in 0.1 M Phosphate buffer, pH 7.4. The homogenate was centrifuged at 4000 rpm for 15 minutes and the supernatant was taken for assays of MDA and GSH.

Procedures of Assays

Assay of Malondialdehyde (MDA)

MDA, the sensitive and convenient marker of lipid peroxidation, was assayed as Thiobarbituric acid-reactive substances(TBARS), by the method of Ohkawa et al.[14] MDA reacts with thiobarbituric acid at 1000c in acidic medium to form pink coloured complex. The colour intensity of MDA-TBA complex is measured at 535 nm. MDA concentration is calculated using the molar extinction coefficient of MDA-TBA complex [$1.56X10^5$ L mol^{-1} cm^{-1}].

To 0.75 ml of the sample (hemolysate/lens homogenate), 3 ml of MDA reagent (75mg thiobarbituric acid + 15gm trichloroacetic acid, in 2.08mL of 0.2N HCl, made up to 100mL with distilled water) is mixed and kept in boiling water bath for 20 minutes. It is then cooled under tap water, centrifuged at 3000 rpm for 10 minutes and absorbance is measured at 535 nm against reagent blank. The level of MDA is calculated using the molar extinction coefficient of MDA-thiobarbituric acid complex.

$$\text{MDA in nanomoles /100 mL} = \frac{\text{OD of sample} \times \text{Total reaction volume}}{\text{Nanomolar Extinction coefficient} \times \text{Sample volume}}$$

$$= \frac{\text{OD of sample} \times 3.75}{1.56 \times 10^{-4} \times 0.75}$$

MDA in nanomoles/100 mL = OD of sample × 3205.1

Assay of Glutathione peroxidase (GSH)

GSH in hemolysates and Lens nucleus was assayed by the method of Beutler et al.[15]

Principle: GSH reduces 5, 5 dithio, bis-nitrobenzoic acid (DTNB) to yellow colored 5-thionitrobenzoic acid (TNB). Absorbance measured at 412nm is directly proportional to the concentration of GSH.

Procedure: To 0.2ml hemolysate/lens homogenate, 1.8ml distilled water and 3ml precipitating solution (metaphosphoric acid + EDTA + NaCl) are added, mixed and kept for 5min and then filtered. To 2 ml filtrate, 8ml phosphate solution (0.3M disodium hydrogen orthophosphate) and 1ml DTNB (40 mg% in 1% sodium citrate) are mixed and absorbance is read at 412nm against the blank. GSH standards ranging concentration from 25 mg/100 ml to 100 mg/100 ml are run simultaneously and GSH concentration in the test sample is calculated from the standard curve. Hb concentration is measured in hemolysates and the GSH level in hemolysates is expressed as micromoles/g Hb.

Statistical Analysis was done using the latest SPSS software. The values were expressed as Mean and Standard Deviation. Significance of the results was evaluated by Student's 't' test. Correlations were done by Karl Pearson's Correlation Analysis.

RESULTS

The nucleus of the lens and the blood samples from 30 diabetics and 30 non-diabetics were assayed for MDA and GSH. The values were subjected to statistical analysis and the results are presented in Tables 1 and 2; Figures 1 to 4.

Tables 1 and 2 show that there was significant increase in MDA levels in lens and blood in diabetic cataract patients. Levels of GSH in lens and blood were significantly lower in diabetic cataract patients.

MDA and GSH in Blood and Lens of Cataract patients

The mean and the standard deviation of the levels of MDA and GSH in blood and lens of the 60 patients were calculated. The 'p value' of less than 0.001 was obtained in each of the 4 categories.

Correlation among the Biochemical Parameters in Cataract Patients

Correlation analysis revealed that MDA in blood and lens showed significant correlation, both in diabetic and non-diabetic cataract. There was a significant correlation among blood and lens GSH levels as well in both the groups of patients. MDA correlated negatively with GSH in all cataract patients.

Positive 'r values' were obtained in the correlation between the levels of MDA in blood and lens as well as the levels of GSH in blood and lens. In contrast, the correlation between the levels of MDA and GSH in blood and lens separately gave negative 'r values' indicating a negative correlation between the two.

Figure 1 shows that the MDA values in blood are in the higher range in Diabetics compared to those of Non-diabetics. Among diabetics, most values are in the range of 81-90 nM/gHb while majority of the values are in the range of 61-70 nM/gHb in non-diabetics.

Figure 2 shows a comparison between the MDA values in Lens among Diabetics and Non-diabetics. The values are higher in diabetics and the commonest range in them is 101-110 nM/dL. Among non-diabetics, most values are in the range 81-90 nM/dL.

Figure 3 shows the GSH values in blood in Diabetics and Non-diabetics. The values are found to be lower among diabetics compared to non-diabetics. It is seen that the commonest range among diabetics is 5.1-5.5 uM/gHb while that in non-diabetics is 7.6-8.0 uM/gHb.

Figure 4 shows a comparison between the GSH values in Lens among Diabetics and Non-diabetics. The values are lower in diabetics and most values are in the range 5.6-6.0 uM/dL while most values are in the range 8.1-8.5 uM/dL in non-diabetics.

Table 1: MDA and GSH levels in Blood and Lens of Cataract patients.

Group	Mean	Std. Deviation	p value
MDA: Blood DIABETIC (nM/gHb) NON-DIABETIC	84.80 60.94	5.845 6.966	$p < 0.001$
MDA: Lens DIABETIC (nM/dL) NON-DIABETIC	104.60 77.45	6.145 7.637	$p < 0.001$
GSH: Blood DIABETIC (uM/gHb) NON-DIABETIC	5.320 7.361	0.5927 0.8535	$p < 0.001$
GSH: Lens DIABETIC (uM/dL) NON-DIABETIC	5.35 7.49	0.690 0.806	$p < 0.001$

Table 2: Correlation of MDA and GSH levels blood and lens with cataract.

			R value
DIABETIC	MDA:blood(nM/gHb)	MDA:Lens (nM/dl) GSH:Blood(Um/gHb)	0.783 -0.933
	MDA:Lens (nM/dl)	GSH:Lens (Um/dl)	-0.606
	GSH:Blood(Um/gHb)	GSH:Lens (Um/dl)	0.964
NON DIABETIC	MDA:blood(nM/gHb)	MDA:Lens (nM/dl) GSH:Blood(Um/gHb)	0.930 -0.833
	MDA: Lens (nM/dl)	GSH:Lens (Um/dl)	-0.830
	GSH:Blood(Um/gHb)	GSH:Lens (Um/dl)	0.965

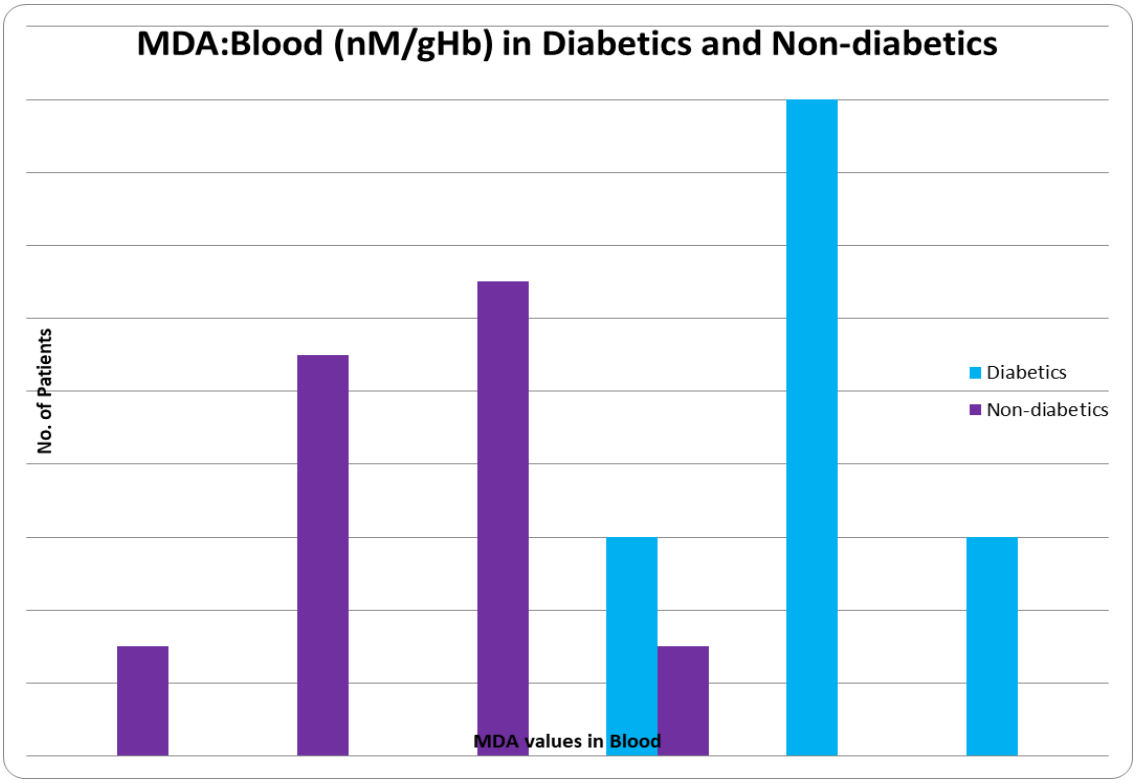

Figure 1: Shows that the MDA values in blood are in the higher range in Diabetics compared to those of Non-diabetics. Among diabetics, most values are in the range of 81-90 nM/gHb while majority of the values are in the range of 61-70 nM/gHb in non-diabetics.

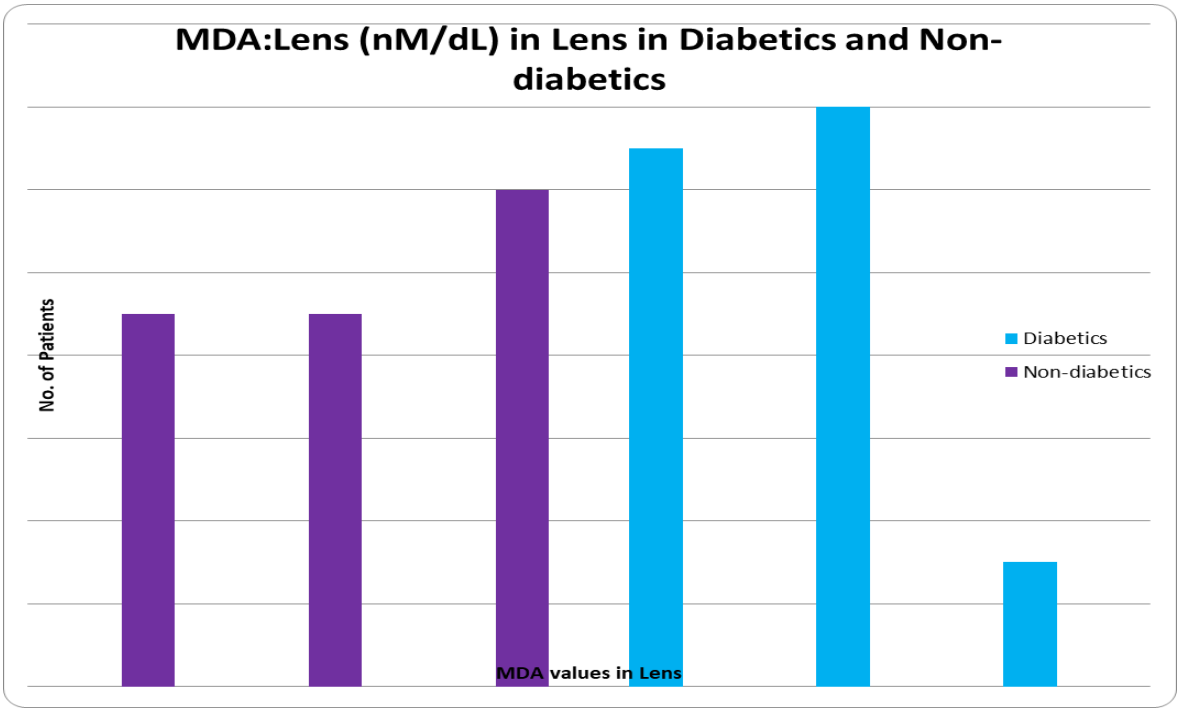

Figure 2: Shows a comparison between the MDA values in Lens among Diabetics and Non-diabetics. The values are higher in diabetics and the commonest range in them is 101-110 nM/dL. Among non-diabetics, most values are in the range 81-90 nM/dL.

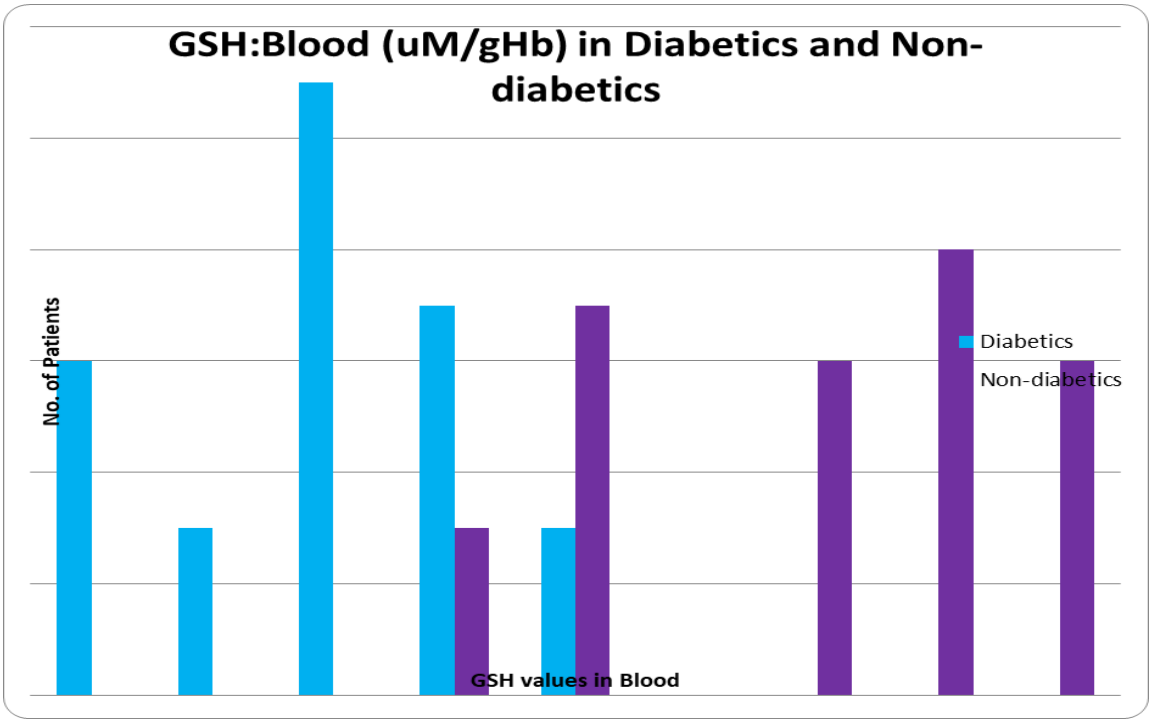

Figure 3: Shows the GSH values in blood in Diabetics and Non-diabetics. The values are found to be lower among diabetics compared to non-diabetics. It is seen that the commonest range among diabetics is 5.1-5.5 uM/gHb while that in non-diabetics is 7.6-8.0 uM/gHb.

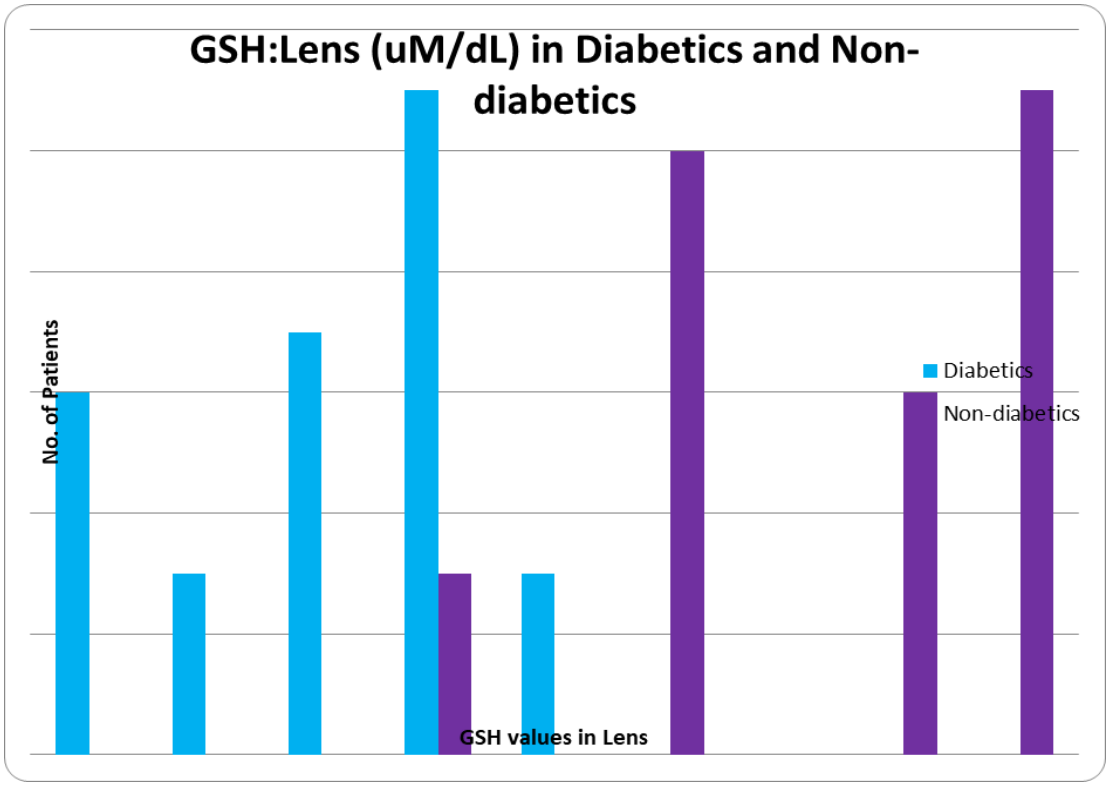

Figure 4: Shows a comparison between the GSH values in Lens among Diabetics and Non-diabetics. The values are lower in diabetics and most values are in the range 5.6-6.0 uM/dL while most values are in the range 8.1-8.5 uM/dL in non-diabetics.

DISCUSSION

Oxidative mechanisms are believed to play an important role in the pathogenesis of cataract formation. Excessively high levels of free radicals cause damage to cellular proteins, membrane lipids and nucleic acids, and eventually cell death. Various mechanisms have been suggested to contribute to the formation of these reactive oxygen free radicals. Glucose oxidation is believed to be the main source of free radicals. The superoxide anion radicals undergo dismutation to hydrogen peroxide, which if not degraded by catalase or glutathione peroxidase, and in the presence of transition metals, can lead to production of extremely reactive hydroxyl radicals. Catalase, glutathione peroxidase (GSH) and superoxide dismutase are considered as key antioxidants that protect the function and the integrity of the lens fibers.

Extensive research has focused on the central role of the enzyme Aldose Reductase (AR) pathway as the initiating factor in diabetic cataract formation. The enzyme AR catalyzes the reduction of glucose to sorbitol through the polyol pathway, a process linked to the development of diabetic cataract. The increased accumulation of sorbitol creates a hyperosmotic effect that results in an infusion of fluid, and this osmotic stress in the lens

caused by sorbitol accumulation induces apoptosis in lens epithelial cells (LEC) leading to the development of cataract.[10]

In a study by Donma et al[11], blood samples and lenses of 46 patients with cataract were studied along with the blood samples of 20 controls. Superoxide dismutase (SOD), glucose-6-phosphate dehydrogenase (G6PD), glutathione reductase (GSSG-Red) activities in red blood cell (RBC) lysates as well as whole blood glutathione (GSH) and Plasma Thiobarbituric Acid Reactive Substances (TBARS), the indicator of lipid peroxidation concentrations, were determined quantitatively both in the blood samples and the lenses of the patients with senile and diabetic cataracts. A strong correlation was found between lens GSH and lens TBARS in the diabetic patients. This also emphasized the vital role of GSH as an antioxidant in the lens over the other antioxidant parameters, e.g., enzymes, and the oxidative stress is at the highest level in lens. Whole blood GSH values and erythrocyte superoxide dismutase (SOD) activities were significantly lower in diabetic cataract patients compared to the senile cataract group. These values were also lower in both these groups compared to controls without cataract. Lens GSH values were also lower in diabetic cataract group compared to the senile cataract group.

Saygili E I et al conducted a study focused on the relation between the oxidative stress and the cataractogenesis especially in diabetes.[12] This group studied the venous blood samples of 75 patients with cataract out of which 25 were type II diabetics. Total oxidant status (TOS), Total antioxidant capacity (TAC) and total free –SH levels were measured in the sera of the patients. TOS and TAC were determined using novel automated measurement methods. With the calculation of Oxidative Stress Index (OSI) using the Total oxidative status and total antioxident capacity, the oxidative status was estimated. Results of the study showed that the Total oxidative status and the oxidative stress were significantly higher in diabetic cataract than in senile cataract and this might also have a role in the patho-physiology of diabetic cataract.

Maurya OP et al studied other important antioxidants like superoxide dismutase and catalase, which protect against free radicals.[13] Estimation of serum levels of superoxide dismutase was done by improved spectrophotometric assay based on epinephrine auto-oxidation at 480nm, while catalase estimation was done by the method of Hugo Aebi in 2 groups of 20 patients each of senile cataract and diabetic cataract.

The mean serum levels of superoxide dismutase and catalase were significantly lower in diabetic cataract patients compared to senile cataract patients without diabetes. This again shows the role of antioxidants in protecting the lens. Studies have shown that this is also true regarding other tissues of the body.

In our study, a total of 60 non smokers with cataract were analyzed for markers of oxidative stress and anti-oxidative activity in the blood and the lens. The number of diabetics (Type II diabetes) and non-diabetics were equal, 30 patients each. It was seen that the average age of the patients with diabetes and cataract was lower than that of the non diabetics.

The blood was assayed for MDA, the sensitive and convenient marker of lipid peroxidation as (TBARS) by method of Ohkawa et al.[14] It showed that the mean values of MDA in blood were significantly higher in diabetics compared to the non-diabetics. This suggests a higher oxidative stress in diabetics when compared to non diabetics. The MDA levels in blood correlate to the MDA levels in the lens in both diabetics and non-diabetics. However the levels of MDA are higher in diabetics in both the lens and in blood. This suggests that the level of oxidative stress in the blood may be reflected in an end organ (cataractous lens in this study) and a higher oxidative stress may be the reason for other end organ damage as suggested in other studies.[16]

The blood samples were assayed for GSH by the method of Beutler et al[15] and it showed that the mean levels of GSH in blood and lens were significantly higher in non-diabetics when compared to diabetics. This finding is in accordance with the findings of a study[17] which found diabetes to be pro-oxidant state. The GSH levels in blood co-relate to the GSH levels in the lens in both diabetics and non-diabetics. The higher GSH levels found in non-diabetics may be the reason for the later onset of cataract. The GSH levels have an inverse correlation with the MDA levels in blood and the lens in both diabetics and non diabetics. GSH, being an antioxidant, is protective against cataract and a decrease in its levels is associated with an increase in the levels of MDA in blood and lens.

This study gives us an idea regarding the levels of the major markers of oxidative stress and anti-oxidative activity in blood and the lens. However cataract is a multi-factorial disease[4], hence it requires further research in finding the role of other oxidative substances responsible for cataract formation. This study indicates the need for further research in oxidative stress in

other end organ damage in diabetics and finding suitable anti-oxidants in preventing / prolonging the onset of end organ damage.

Due to limited number of patients included in this study, the type of cataract and the onset of cataract could not be correlated to the level of oxidative stress. It is hypothesized that an imbalance between oxidant and antioxidant status is one of the key factors in the development of cataract especially in diabetic subjects.

In this observational study, we tried to look into the oxidant-antioxidant levels in the blood and lens of 30 willing cataract patients with diabetes and a similar number of cataract patients without diabetes who presented themselves in the OPD of the department of Ophthalmology.

Malondialdehyde (MDA), a sensitive marker of lipid peroxidation as the oxidant and Glutathione (GSH) as a strong antioxidant were measured as described in the procedure.

The MDA levels in blood correlate to the MDA levels in the lens in both diabetics and non-diabetics. The mean value of MDA in the diabetic cataract patients was found to be significantly higher than that in non-diabetic patients. Among diabetics, most values in the blood were in the range of 81-90 nM/gHb while in non-diabetics, majority of the values were in the range of 61-70 nM/gHb. Similarly The values in the lens were found to be higher in diabetics in the range of 101-110 nM/dL, where as the same in non-diabetics were in the range 81-90 nM/dL.

The mean levels of protective GSH in the blood and lens were significantly higher in non-diabetics. The range of values in the blood was 7.6-8.0 uM/gHb and in the lens it was 8.1-8.5 uM/dL. The ranges in the diabetic patients in the blood and lens were 5.1-5.5 uM/gHb and 5.6-6.0 uM/dL respectively which were significantly lower than in non diabetic patients.
This study showed that the levels of oxidants are higher and that of antioxidants are lower in diabetic cataract patients suggesting a higher oxidative stress in diabetic patients. However, there is a larger scope for further research with a larger number of patients/volunteers to find out more about the adverse effects of the oxidants and the beneficial effects of antioxidants, especially in the form of medications to prevent the end organ damage like cataractogenesis.

CONCLUSION

MDA and GSH are the major markers of oxidative and anti-oxidative activity respectively in the blood and lens. The increased oxidative stress in the lens of diabetics may be primarily

responsible for cataract formation in them at a younger age compared to non diabetics. The end organ damage in diabetics may be a result of increased oxidative stress in the blood. Hence this study indicates further research in this regard.

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