Eales' Disease: Identification, Purification and Characterization of a New 88 kDa Glycoprotein and the Role of Oxidative Stress in the Pathogenesis of Retinal Inflammation and Neovascularization

THESIS

Submitted in partial fulfillment

of the requirements for the degree of

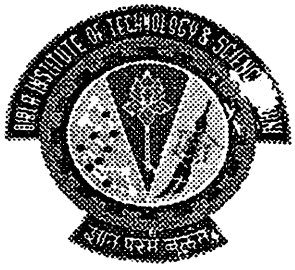
DOCTOR OF PHILOSOPHY

By

M. RAJESH

Under the supervision of

Dr. S. RAMAKRISHNAN



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Dedicated to
Dr.K.N. Sulochana

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CERTIFICATE

This is to certify that the thesis entitled "***Eales' Disease: Identification, Purification and Characterization of a New 88 kDa Glycoprotein and the Role of Oxidative Stress in the Pathogenesis of Retinal Inflammation and Neovascularization***" and submitted by **Mr. M. Rajesh, ID. No. 2000PHXF005** for award of Ph.D. Degree of the Institute, embodies original work done by him under my supervision.

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CONTENTS

LIST OF ABBREVIATIONS

APR	Acute phase reactant
AV	Active vasculitis
BCIP	5- Bromo – 4 – chloro 3- indoyl phosphate
BRB	Blood – retinal- barrier
BSA	Bovine serum albumin
CH_3CN	Methyl cyanide (acetonitrile)
CH_3OH	Methanol
cDNA	Complementary Deoxyribonucleic acid
DAB	3,3'diamino benzidine
2dG	Deoxyguanosine
DEAE	Diethylaminoehtyl cellulose
dH2O	Distilled water
DNA	Deoxyribonucleic acid
DNPH	2,4-dinotrophenyl hydrazine
DTNB	5,5'dithiobisnitrobenzoic acid
DTPA	Diethylenetriaminepenta acetic acid
DTT	Dithiothreitol
ED	Eales' disease
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial nitric oxide synthase
ERM	Epiretinal membrane
ESR	Electron spin resonance spectrometer
$FeCl_3$	Ferric chloride
GC-MS	Gas chromatography- Mass spectrometer
GCL	Ganglion cell layer
G6PD	Glucose 6 phosphate dehydrogenase
GPx	Glutathione peroxidase
GSH	Glutathione

H_2O_2	Hydrogen peroxide
H_3PO_4	Phosphoric acid
Hb	Hemoglobin
HCl	Hydrochloric acid
HBSS	Hanks balanced salt solution
HLA	Human leukocyte antigen
HOCl	Hypochlorous acid
Hp	Haptoglobin
Hpx	Hemopexin
HV	Healed vasculitis
ICAM	Intracellular adhesion molecule
IL	Interleukin
INL	Inner nuclear layer
iNOS	Inducible nitric oxide synthase
MC	Monocytes
MDA	Malondialdehyde
$MgCl_2$	Magnesium chloride
MMP	Matrix metalloproteineases
MPO	Myeloperoxidase
MT	Metallothioneins
NaI	Sodium iodide
NaOH	Sodium Hydroxide
NBT	Nitrotetrazolium blue chloride
nNOS	Neuronal nitric oxide synthase
NPAGE	Native polyacrylamide gel electrophoresis
3NTYR	3 Nitrotyrosine
NV	Neovascularization
OFL	Outer fiber layer
ONL	Outer nuclear layer
OPL	Outer plexiform layer
$O_2^{\bullet -}$	Superoxide anion

$OH^{\bullet}$	Hydroxyl free radical
8-OHdG	8 – hydroxy 2'-deoxyguanosine
$ONOO^{-}$	Peroxynitrite
PAS	Periodic acid Schiff stain
PBS	Phosphate buffered saline
PCMB	Parachloromercuric benzoate
PMA	4α - phorbol – 12- myristate 13 – acetate
POBN	4-pyridyl-1-oxide N tert butyl nitrone
PUFA	Polyunsaturated fatty acid
PVDF	Polyvinylidine difluoride membrane
RBCs	Red blood cells
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RP HPLC	Reverse Phase High Performance Liquid Chromatography
RPE	Retinal pigment epithelial cells
RRTF	Redox regulated transcription factors.
RT	Room temperature
RV	Retinal vasculitis
SDS PAGE	Sodium dodecyl polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
Tf	Transferrin
TFA	Trifluoroacetic acetic acid
Trx	Thioredoxins
VEGF	Vascular endothelial growth factor
VH	Vitreous hemorrhage
XOD	Xanthine oxidase

INTRODUCTION

Introduction

The retina forms the inside lining of the back of the eye and is composed of light-sensitive neurons. The retina processes the light emitted from visual images via transduction (the conversion of energy from one form to another), and transmits this information to the brain for perceptual awareness of the images. The light must pass through all layers of the retina before it reaches the cells that systematically absorb the light energy.

In more detailed descriptions of the retina, the three primary retinal layers are further divided into seven layers. For example, in one seven layer organization the following are distinguished: dark "nuclear/cell" layers containing cell bodies and white "plexiform" layers containing axons and dendrites. The first layer contains the pigment end of the receptor cells. The second, outer nuclear layer (ONL) contains cell bodies of the receptor cells. The third, outer plexiform layer (OPL) contains the dendrites and axons of the receptor, horizontal, and bipolar cells. The fourth, inner nuclear layer (INL) contains the dendrites and axons of the horizontal, bipolar, and amacrine cells. The fifth, ganglion cell layer (GCL) contains the cell bodies of ganglion cells. Finally, the optic fiber layer (OFL) contains the axons of ganglion cells as they collect to form the optic nerve. The retinal pigment epithelium, which lines the back of the eyeball behind the receptor cells, is composed of a single layer of cells. This maintains protects neural retina from circulating blood and provides tight junction, which serves as blood-retinal-barrier [BRB] (Gorden and Bazen, 1997). The retina is comprised of microvessels, which supplies blood and provides oxygen to the retinal tissue. During certain pathological conditions, either the inflammation or occlusion in these blood vessels results in the condition called as

ischemia (Iampal et al., 1994). Ischemia has been shown to trigger the expression and release of certain growth factors and proteases, which aid in the process, called as retinal neovascularization (new vessel formation) by the disruption of BRB (Stitt, 2001). The new vessels formed are fragile and they rupture resulting in vitreous hemorrhage (VH) and retinal detachment (RD). All these eventually result in the loss of vision (Iampal et al., 1994). There are several systemic diseases, where retina is affected for example, diabetes (diabetic retinopathy), sickle cell anemia (sickle cell retinopathy), sarcoidosis (retinal vaculitis), tuberculosis (retinal vasculitis) etc., (Iampal et al., 1994).

Eales' disease (ED) is an idiopathic inflammatory venous occlusion, which primarily affects the peripheral retina of young adults (Eales 1880 & 1882). ED is rare in Western and South East Countries, but for unknown reasons it is common in the Indian subcontinent, with the incidence of 1 in 300 ophthalmic patients evaluated in a tertiary eye care center (Puttanna, 1970). ED predominantly (99 %) affects males in the age group of 20-45 years and is often bilateral (Puttanna, 1970).

The clinical features include perivascular phlebitis, non-perfusion and neovascularization (NV). Bleeding from NV is common, usually recurrent and is one of the major causes for visual loss in patients with ED (Das et al., 1994). In addition to the above, the formation of epiretinal membrane (ERM) imposes a tractional pull on the retina causing retinal detachment. Timely intervention with vitrectomy, laser photocoagulation or removal of epiretinal membrane helps in partial restoration of vision. Treatment for ED is mainly based on the symptoms. Often the management of intraocular inflammation is done using

oral corticosteroids. Vitreoretinal complications are treated with either laser photocoagulation or other vitreoretinal surgical procedures (Das et al., 1994 ; Biswas et al., 2002).

Despite rigorous research at various centers in the country, the etiology of ED remains unknown. Several biochemical and Immunologiccal studies have yielded inconclusive results. Acute phase reactants (APR) are group of glycoproteins, expressed to have specific functions during pathological conditions. Most often decreased or increased levels of APR in circulation are used as presumptive diagnostic markers. In certain hematological diseases, haptoglobin (Hp) a 65 kDa glycoprotein is synthesized by liver and secreted in to circulation to scavenge the hemoglobin (Hb) and prevent the loss of iron from the body, thus help in regulating iron metabolism (Halliwell and Gutteridge, 1990). Therefore identifying novel APR, which are expressed in body fluids / local tissue during inflammatory conditions might help us to understand the pathomechanism of disease process and could probably be used as diagnostic / prognostic marker

Rengarajan et al., (1989) have identified and purified a 23 kDa protein from serum of patients with ED and they speculate that this protein might be involved in the pathogenesis of ED. Bora et al., (1996) have reported the presence of 36 kDa protein in serum of patients with parsplanitis. However sequencing of cDNA for 36 kDa protein revealed it is homologous to the yeast nuclear pore complex protein, speculating the role of this protein in the pathogenesis of parsplanitis. However, there are no such reports available in ED.

The present study describes the identification, purification and characterization of a new 88 kDa protein found in serum and vitreous of patients with ED. Based on the physiochemical properties, possible reasons for its expression in ED are discussed. Since, 88 kDa protein was predicted to belong to the family of APR proteins, further investigations were done to identify its expression in other ocular inflammatory and systemic diseases, and the results are presented chronologically.

Oxidative stress has been shown to play an important role in the pathogenesis of several human inflammatory diseases, including ocular inflammatory diseases (Cross et al., 1987; Rose et al., 1998). Antioxidant supplementation has been shown to ameliorate the harmful effects of oxidative stress and delay the progression of several human diseases, wherein the role of oxidative stress in the disease process is well established (Cuzzocrea et al., 2001). Moreover, inflammation, angiogenesis and oxidative stress are shown to be interdependent in several human diseases (Jackson et al., 1997).

In ED inflammation and neovascularization are the main clinical manifestations (Spitzans et al., 1975). Preliminary studies have revealed diminished levels of antioxidant enzymes and vitamins in erythrocytes and vitreous obtained from patients with ED (Bhooma et al. 1997 ; Sulochana et al., 1999). In the present study, further investigations were performed to understand the role of oxidative stress in the development of retinal vasculitis and neovascularization in ED by probing the role of phagocyte generated hydroxyl radical and reactive nitrogen species. In addition to the above, the extent of oxidative protein and DNA damage has been investigated for the first time in Eales' disease.

AIMS
&
OBJECTIVES

AIM & OBJECTIVES

Hypothesis

As oxidative stress, inflammation and angiogenesis are proven to be co-dependent, oxidative stress was suspected to be associated with the disease process in ED. Identifying the expression of novel protein could provide insights in understanding the mechanism behind the development of retinal vasculitis and neovascularization in patients with ED.

Aim

The scope of the present study was to investigate the role of oxidative stress in promoting RV and NV in ED. To identify and characterize a novel protein expressed in ED.

Specific Objectives

Understand the role of oxidative stress in the development of RV and NV in ED by

1. Determining the production of hydroxyl free radical ($OH^{\bullet}$) generation in monocytes (MC) by using ESR technique.
2. Investigating the role of inducible nitric oxide synthase (iNOS) expression and reactive nitrogen species (RNS) mediated tissue damage in ED.
3. Determining the extent of oxidative damage to proteins by determining the levels of carbonyl groups formation in plasma.

4. Assessing the oxidative DNA damage in leukocytes of patients with ED by determining DNA adduct – 8 - hydroxy 2'- deoxyguanosine (8OHdG) using Gas Chromatography/ Mass Spectrometer (GC/MS) technique in leukocytes.

Study the role of a novel protein expressed in ED by

1. Identifying the expression of any novel protein in serum and vitreous samples obtained from patients with ED.
2. Developing purification techniques to isolate and purify the protein to homogeneity.
3. Characterizing the physical and chemical properties of the purified protein and identify the biological function of this protein.
4. Investigating the expression of the novel protein in other intraocular inflammatory diseases and systemic diseases and again look for the immunological identity, if the protein is present in other diseases.

REVIEW OF LITERATURE

Review of Literature

Henry Eales a British ophthalmologist in 1880 and 1882 described the series of clinical picture with recurrent retinal haemorrhages in young adults. The patients were young men aged 14 to 29 years. They had common history of headache, epistaxis, and variation in peripheral circulation, dyspepsia and chronic constipation. Eales believed it to be vasomotor necrosis wherein constriction of the alimentary vessels and compensatory dilatation lead to rupture of retinal vessels with subsequent haemorrhage. However, retinal vasculitis (RV) was not described by Eales. Five years later Wadsworth (1887) described the associated signs of retinal inflammation. In the century that has followed, Henry Eales has been honored with eponym for the disease characterized by idiopathic recurrent vitreous hemorrhage (VH) in young adult males. Elliot (1948) initially suggested that the disease should be called as "periphlebitis retinae". However, equal prevalence of vascular and arteriolar inflammation was later documented by (Kimura et al., 1956) since then ED was entitled as one of the RV condition.

Clinical Manifestations

Patients are often asymptomatic in the initial stages of retinal perivasculitis. Some patients present with symptoms such as floaters, blurring of vision or gross diminution of vision due to massive VH. Vision in these patients can be hand movements or light perception only. Bilaterality is common in patients with ED. Clinical manifestation of ED is due to three pathological changes: inflammation, ischemia and NV in the retina or disk, which leads to vitreous hemorrhage. Signs and symptoms of inflammation occur at varying times in the course of the disease, but are less common in later stages. Fundus

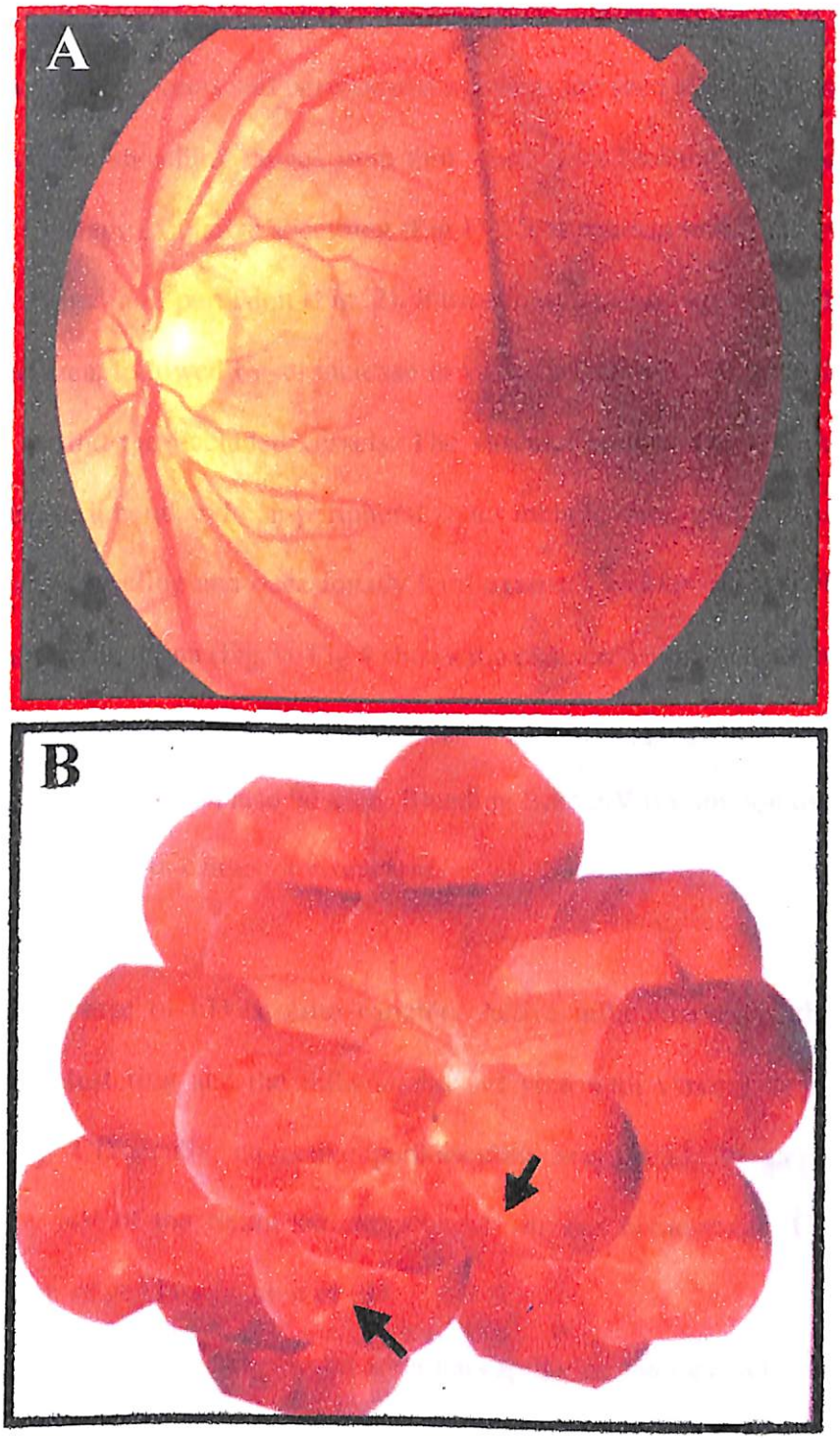


Figure 1: (A) Fundus picture of an healthy individual. (B) Montage fundus photograph of a patient with Eales' disease. Active inflammation of the retinal veins are denoted by arrow

examination in the early stage of the disease reveals venous dilatation in the periphery with discontinuity of veins and perivascular exudates are seen along the peripheral veins (Fig.1). Vascular sheathing ranges from thin white lines limiting the blood column on both sides to heavy exudative sheathing (Fig.1). Most patients with ED develop varying degrees of retinal non-perfusion (Fig. 2). Intraretinal haemorrhages often first appear in the affected area, followed by an increase in vascular tortuosity with frequent collateral formation around the occluded vessels. The vascular abnormalities at these junctions between the peripheral and non-peripheral zones include microaneursyms, veno-venous shunts, venous beading and occasionally hard exudates and cotton - wool spots. NV is usually seen in the retina (Fig.3). Fig.4 shows the characteristic NV pattern in ED. Fig. 5 illustrates the formation of scar membrane during the disease progression. However in some cases NV at disc can also be seen. Bleeding from NV is common usually recurrent and is one of the major causes of visual loss.

The natural course of ED is quite variable. Active inflammation is characterized by perivascular clustering and the inflammation of vein with varying degrees of venous insufficiency. Classically, an active perivasculitis stage leads to an ischemic stage followed by NV of the retina and subsequent vitreous hemorrhage. Charmis, (1965) proposed four stage classification of ED.

Stage 1: mild periphlebitis of peripheral retinal capillaries, arteries and veins.

Stage 2: perivasculitis of the venous capillary widespread and larger veins being affected with vitreous haze.

Stage 3: new vessel formation with abundant hemorrhage in the retina and vitreous.

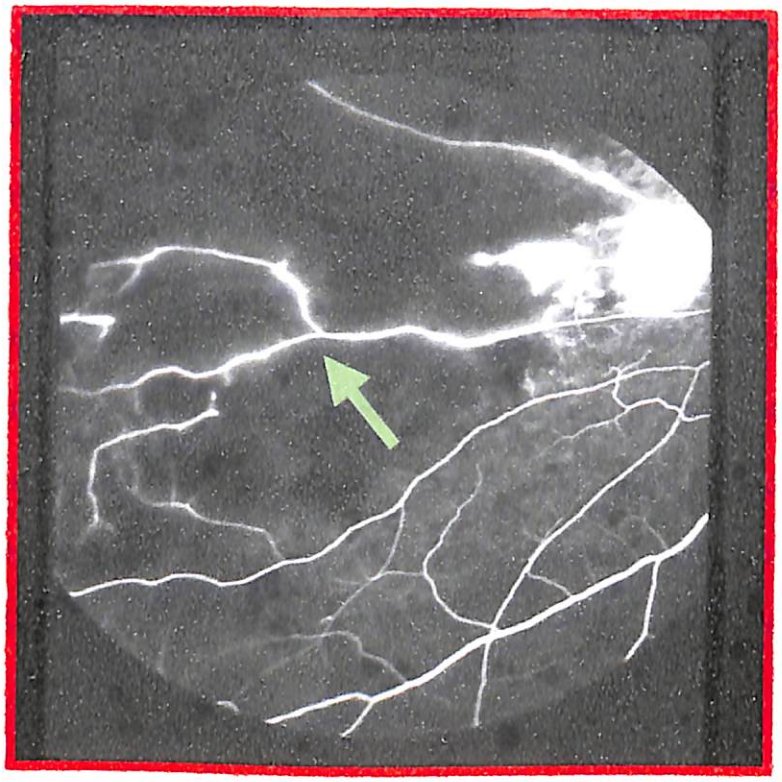


Figure 2. Fundus picture of a patients with ED, showing active vasculitis with ischemic changes in the retinal veins denoted by arrow

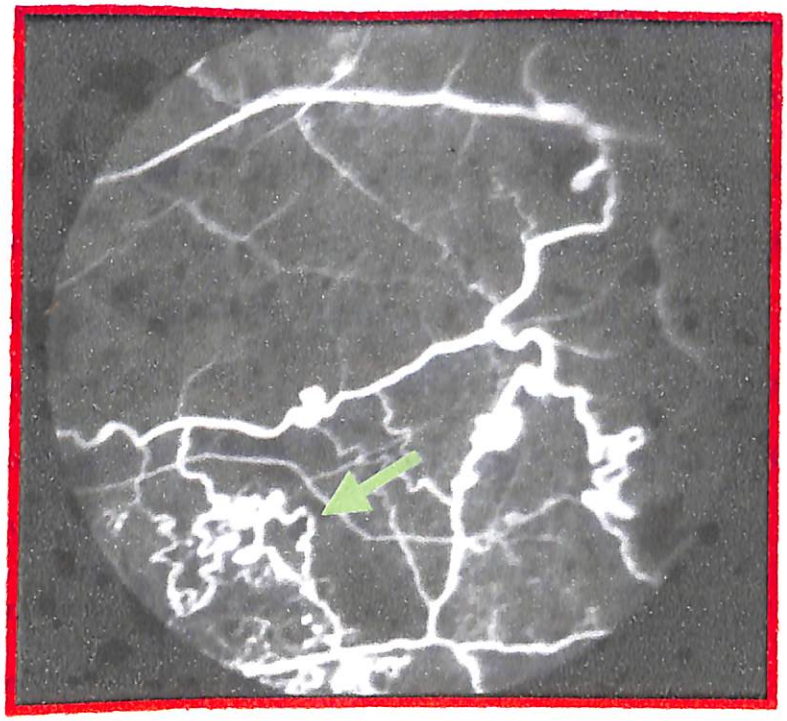


Figure 3. Fluorescein fundus angiogram of a patient with ED, showing characteristic veno-venous shunts.

Stage 4: the advanced stage is characterized by massive and recurrent vitreous hemorrhage with retinitis proliferans and tractional retinal detachment (RD). Das and Namperumalswamy, (1987) have proposed a different system of grading for ED, based on the degree and extent of microangiopathy, proliferative retinopathy and VH. However, the four-stage classification and the above-described classification are not popular due to the overlapping stages in the clinical manifestation. Yet, till date no standard system for grading has been accepted and practiced. In spite of extensive intraocular inflammation and long-standing VH, the lens remains clear. As the disease presents in various stages to the clinician several modalities of treatment are suggested based on the clinical symptoms.

The aetiopathogenesis of ED to date remains controversial and poorly understood and is considered to be idiopathic. Duke Elder (1987) felt that ED is not a specific entity but is a manifestation of many diseases. However now ED is considered to be a specific clinical entity. RV and peripheral retinal NV associated with various systemic and ocular diseases can mimic ED. Therefore ED should be differentially diagnosed from several other retinal vasculitis conditions. RV mimicking ED is listed in Table 1, while proliferative retinopathy mimicking ED is listed in Table 2. The aetiopathogenesis of ED remains obscure since its description, various efforts have been taken to identity the etiology, by studying the:

1. Systemic diseases association.
2. Immunological status.
3. Biochemical abnormalities.

Figure.4

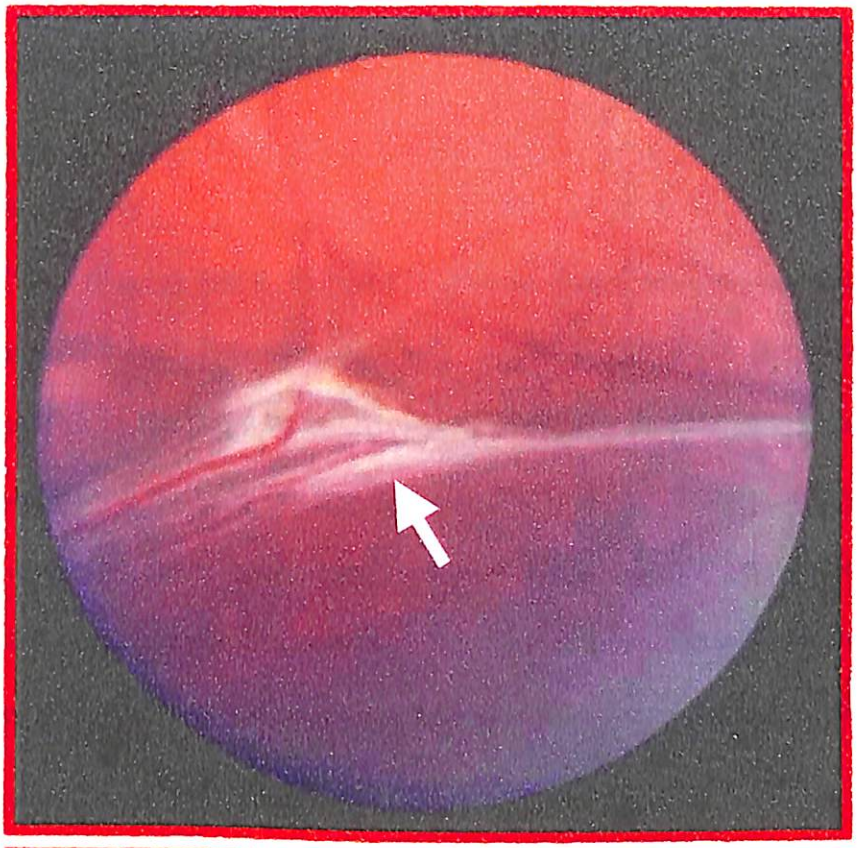


Figure.5

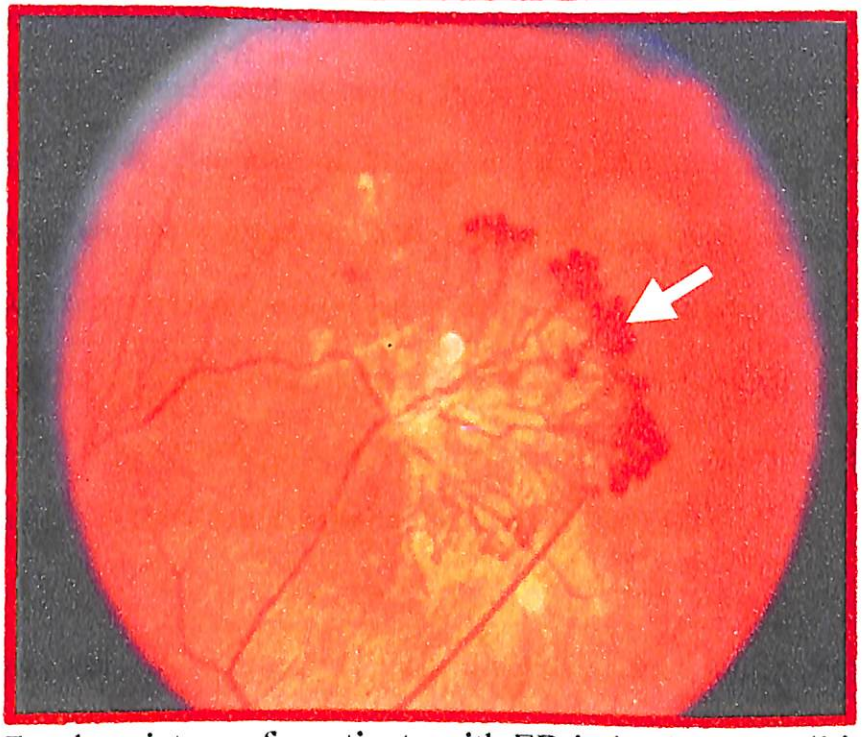


Figure 4. Fundus picture of a patients with ED in healed vasculitis stage, presenting with whitish fibrovascular sheahing. **Figure 5**. Fundus picture of a patient with ED, where characteristic sea fan noevascularization could be seen.

4. Histopathological characteristics.

Systemic diseases

In 1880 Henry Eales first described ED in detail. Later, several systemic diseases have been associated with ED (Das et al., 1994). However, many such associations have been found to be sporadic. But tuberculosis has been directly or indirectly associated with ED (Reviewed by Das et al., 1994 ; Biswas et al., 2002). Based on the possible association of tuberculosis with ED, Finoff, (1921) injected tubercle bacilli into homolateral carotid artery of 50 rats. However retinal vascular changes were observed in only one animal. Subsequently several investigators were not able to get the reproducible results.

In a large-scale study, when 1010 patients with pulmonary and extra pulmonary tuberculosis were examined for the presence ED. None of them had ED (Biswas and Badrinath, 1995). Recently Madhavan et al., (2000) reported the presence of *Mycobacterium tuberculosis* DNA in the ERM obtained from patients with ED. However vitreous culture turned out to be negative for *Mycobacterium tuberculosis.* Therefore the authors hypothesize that some antigens of *Mycobacterium tuberculosis* might have been sequestered in the retina, under certain conditions, these antigens evoke an immune response, which could result in the development of RV in ED.

Apart from tuberculosis, some investigators have considered allergic reaction to tubercle protein. Mantoux positivity has been reported in 40-90% of patients with ED (Elliot, 1954). In a recent case-controlled study of ED in India, no statistical significant

Table 1: Retinal vasculitis mimicking Eales' disease

Systemic	Ocular
Behcet's disease	Coat's disease
Multiple sclerosis	Pars planitis
Sarcoidosis	Viral retinitis
Syphilis	
Systemic lupus erythematosis	
Toxocariosis	
Wegner's granulomtosis	

Table 2: Proliferative vascular retinopathy mimicking Eales' disease

Systemic	Ocular
Diabetic Retinopathy	Branch retinal vein occlusion
Sarcoidosis	Central retinal vein occlusion
Sickle cell disease	Pars planitis , Coat's disease

difference was seen in Mantoux positivity. Moreover Mantoux positively was seen in 70-90% of healthy subjects and ED was also seen among Mantoux negative patients (Narain et al. 1975). Evaluation of lymphocyte proliferation to purified protein derivative showed no significant changes between disease and control groups. All these results raised the question on the association of tuberculosis with ED.

Immunological Studies

Jhonson et al., (1969) has reported elevated levels of serum IgG and IgA in patients with ED. Muthukarupan et al., (1989) found normal T lymphocyte subsets and optimal antibody response to retinal S antigen. Recently Biswas et al., (1998) have found higher phenotypic frequencies of human leukocyte antigen (HLA) B51, DR 1 and DR 4 to be associated with ED.

Biochemical studies

Sen et al., (1990) have reported raised levels of serum α1 acid glycoprotein in patients with ED. Recently Swami et al., (2002) have documented the accumulation of advanced oxidation protein products in ERM and serum of patients with ED. Rengarajan et al., (1989) has identified a distinct protein with apparent molecular weight of 23 kDa in serum of patients with ED. The authors speculate that this protein might play a role in the pathogenesis of ED. Since its description in 1989, there are no reports available to date on what is the exact role of this protein in ED? Oxidative stress, inflammation and angiogenesis have been shown to be interdependent in several chronic inflammatory and malignant diseases (Jackson et al., 1997). Based on this fact, Bhooma et al., (1997)

investigated the levels of antioxidant vitamins and levels of thiobarbituric acid reactive substances (TBARS) in erythrocytes of patients with ED. They found decreased levels of vitamin A, E and C in serum and TBARS in erythrocytes of patients with ED, when compared with healthy control subjects. Subsequently, Sulochana et al., (1999) reported diminished activity of superoxide dismutase (SOD), glutathione peroxidase (GPx) and increased accumulation of TBARS in vitreous of patients with ED.

Histopathological studies

Evaluation of epiretinal membrane (ERM) from patients with ED revealed massive infiltration of chronic inflammatory cells. Immuno typing of these cells revealed that they were T cells and monocytes (activated macrophages). The absence of polymorphonuclear cells indicates that the inflammatory tissue damage is chronic in nature (Badrinath et al., 1992). In addition to the above formation of neovascular channels were also observed (Kono et al., 1985 ; Badrinath et al., 1992). These findings suggest that leukocytes play an important role in the development of RV in ED.

Differential diagnosis of ED

Since ED mimic several other RV conditions, it is necessary to diagnose ED more accurately. The routine package laboratory tests requested for diagnosis of ED include hematological, biochemical and serological investigations. The list of the parameters investigated is listed in Table 3.

Table 3: Diagnostic tests performed for differential diagnosis of Eales' disease

1	Routine Tests	
		Complete Haemogram
		Erythrocyte sedimentation rate
		Blood glucose
		Blood chemistry
		Chest Radiograph
		Urine analysis
		C reactive protein
		Sickle cell disease screening test
2	Infectious Diseases	
		VDRL
		TPHA
		Mantoux test
		Brucella agglutination test
3	Autoimmune disorders	
		Antinuclear antibody
		Anti- double stranded DNA antibodies
4	Miscellaneous Tests	
		Serum lysozyme assay
		Serum angiotensin converting enzyme

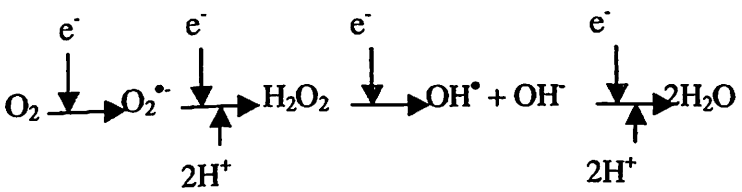
VDRL, venereal disease research laboratory; TPHA, *Treponema pallidum* hemeagglutination test.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS)

A free radical is defined as "any chemical species capable of independent existence that possesses one or more unpaired electrons". In order to understand the role of reactive oxygen species (ROS) and nitrogen species (RNS) in the disease process, one has to know the basic chemistry, factors that aid in their formation and removal.

ROS includes a number of chemically reactive molecules derived from oxygen. Some of those molecules are extremely reactive such as hydroxyl radical ($OH^{\bullet}$), while some are less reactive like superoxide ($O_2^{\bullet -}$). Free radicals have very low half-life and they can react with most of the biomolecules, starting a chain reaction of free radical formation. In order to stop this reaction, a newly formed radical must either react with another free radical, eliminating the unpaired electrons, or react with a free radical scavenger- a chain breaking or primary antioxidant (Finkel et al., 2000; Firdovich 1999).

Table 4 shows most important ROS, with their main cellular sources of production and the relevant endogenous antioxidant systems for scavenging these ROS molecules. The step wise reduction of molecular oxygen via 1-electron transfers, producing ROS are shown in the following reaction:



Reaction 1

Table 4: The major ROS molecules and their metabolism

ROS	Main sources	Defence system
Superoxide ($O_2^{\bullet -}$)	• Leakage of electrons from the electron transport chain. • Activated phagocytes • Xanthine oxidase • Flavoenzymes	• Superoxide dismutase
Hydroxyl radical ($\dot{O}H^{\bullet}$)	• From $O_2^{\bullet -}$ and H_2O_2. • Myeloperoxidase of Phagocytes.	Not Known

At the intracellular level, the ROS formation and metabolism are summarized in Fig. 6. The metabolic pathways and the cellular effects of the ROS are described below.

Superoxide ($O_2^{\bullet-}$)

The superoxide anion produced from molecular oxygen by the addition of an electron. The formation of $O_2^{\bullet-}$ takes place simultaneously , especially in the electron-rich aerobic environment in vicinity of the inner mitochondrial membrane with the respiratory chain (Fig. 6). $O_2^{\bullet-}$ as well as hydrogen peroxide is also produced endogenously by flavoenzymes e.g. xanthine oxidase (Kuppusamy et al., 1989) activated in ischemia-reperfusion conditions (Zimmerman et al. 1994). Other $O_2^{\bullet-}$ producing enzymes are lipoxygenase and cycloxygenase (Kontos et al., 1985). The NADPH-dependent oxidase of phagocytic cells, a membrane-associated enzyme complex constitutes an example for high levels of $O_2^{\bullet-}$ production. Enzymes similar to components of this complex are also present in non-phagocytic cells where their functions are connected with cellular signalling mechanisms (Thannickal and Fanburg, 2000). Two molecules of $O_2^{\bullet-}$ rapidly dismutate to hydrogen peroxide and molecular oxygen and this reaction is further accelerated by SOD.

$$2\,O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \qquad \text{Reaction 2}$$

Hydrogen peroxide (H_2O_2)

Hydrogen peroxide by it self, is not a free radical but is nonetheless highly important because (A) of its ability to penetrate biological membranes. (B) It plays an important role in the production of more reactive ROS molecules such as hypochlorous acid

Figure 6

Schematic representation of oxidative and antioxidant defence systems in cells. Superoxide is produced in significant amounts intracellularly in the cytosol via flavin-containing enzymes and in mitochondria, mainly due to escape of electrons from the respiratory chain. Two molecules of superoxide rapidly dismutate, either spontaneously or via superoxide dismutase to dioxygen and hydrogen peroxide. The latter permitting flux of ROS between cellular compartments. Hydrogen peroxide can be enzymatically metabolized to dioxygen and water by number of different enzyme systems or converted to the hydroxyl radical ($OH^{\bullet}$), which is extremely reactive. They react with almost any biomolecule and oxidize it. The prominent being protein oxidation, DNA oxidation and lipid peroxidation.

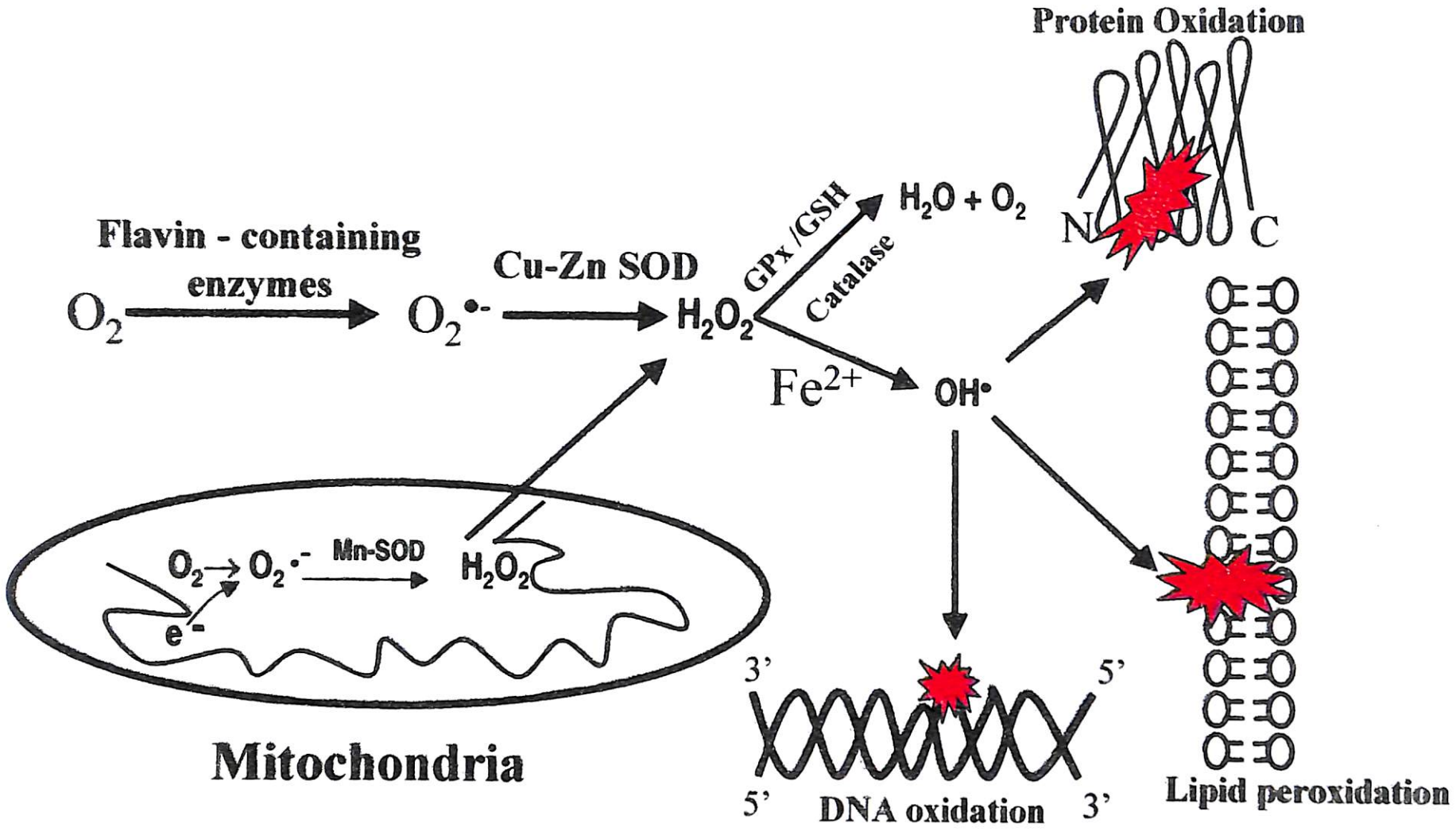


Figure 6. Formation of ROS and its deleterious effects on biomolecules

(HOCl) and hydroxyl [reaction 3,4] by the action of myeloperoxidase (MPO), an enzyme present in the phagosomes of phagocytes (Winterbrown et al., 2000).

$$H^+ + Cl^- + H_2O_2 \rightarrow HOCl + H_2O$$

Reaction 3

Another important function of H_2O_2 is carried out in its role as an intracellular signalling molecule (Rhee., 1999). H_2O_2 once produced by the above mentioned mechanisms is removed by atleast three antioxidant enzyme systems namely, catalase, glutathione peroxidase and peroxiredoxins (Mates et al., 1999) as summarized in Fig.6.

Hydroxyl radical ($OH^\bullet$)

Due to its strong reactivity with biomolecules, $OH^\bullet$ is capable of doing more damage to biological systems than any other ROS (Halliwell, 1985). The half-life of $OH^\bullet$ has been estimated to be 10^{-9} sec under *in vivo* conditions (Firdovich, 1978). The $OH^\bullet$ radical is formed from H_2O_2 in a reaction catalyzed by metal ions (Fe^{2+} or Cu^+), often bound in complex with different proteins or other molecules. This reaction is known as the Fenton reaction.

$$H_2O_2 + Fe^{2+} / Cu^+ \rightarrow OH^\bullet + OH^- + Fe^{3+} / Cu^{2+}$$

Reaction 4

$O_2^{\bullet-}$ also plays an important role in the Reaction 4 by recycling the metal ions:

$$Cu^{2+} / Fe^{3+} + O_2^{\bullet-} \rightarrow Cu^+ / Fe^{2+} + O_2$$

Reaction 5

The sum of Reactions 4 and 5 is the Haber-Weiss reaction; transition metals thus play an important role in the formation of hydroxyl radical (Halliwell, 1999). Transition metals may be released from proteins such as ferritin (Harris et al., 1994) and the iron-sulfur clusters of different dehydrases by reactions with $O_2^{\bullet-}$. This mechanism, specific for living cells, has been called the in vivo Haber-Weiss reaction (Firdovich, 1997).

Physiological Functions of ROS

Defense against infection

When phagocytes are activated, they produce ROS in high amounts to kill intruding microorganisms (Thomas et al., 1988). During this process, ROS are produced by the NADPH oxidase complex that converts O_2 to $O_2^{\bullet-}$ (Babior, 1999 ; Nauseef, 1994). $O_2^{\bullet-}$ is then reduced in the phagosome by SOD to H_2O_2 that can be further converted to HOCl by MPO (Rossi et al., 1985). HOCl may then spontaneously form $OH^{\bullet}$ by the following Reaction 6 or 7. Reaction 7 is analogous to the Fenton reaction (Reaction 4) but with HOCl taking part in the place of H_2O_2.

$$HOCl + O_2^{\bullet-} \rightarrow OH^{\bullet} + O_2 + Cl^- \qquad \text{Reaction 6}$$

$$HOCl + Cu^+ / Fe^{2+} \rightarrow OH^{\bullet} + Cl^- + Cu^{2+} / Fe^{3+} \qquad \text{Reaction 7}$$

The two highly reactive ROS molecules thereby formed in phagosomes (HOCl and $OH^{\bullet}$) are highly toxic to bacteria ingested by the phagocyte and carry out the direct antimicrobial effects of ROS. The HOCl produced in the myeloperoxidase (MPO)

reaction is also an important part of antimicrobial defense by destruction of the DNA anchoring at the bacterial membrane resulting in the cessation of DNA replication (Rosen et al., 1990).

Redox regulation of transcription factor activity

ROS can directly affect the conformation and / or activities of all sulfhydryl-containing molecules such as proteins, by oxidation of their thiol moiety. This type of redox regulation affects many proteins important in signal transduction and carcinogenesis, such as protein kinase C, Ca^{2+} ATPase, collagenase and tyrosine kinase (Dalton et al., 1999), and many other enzymes and membrane receptors (Babior, 1997). For several transcription factors, ROS function as physiological mediators of transcription control (Morel, 1999; Allen 2000). Well –known examples of redox-sensitive transcription factors are Nuclear factor -κB (NF-κB) and Activator protein – 1 (AP-1) (Nakamura, 1997 and Schreck, 1992).

In the case of AP-1 a dimer of gene products from the proto-oncogene families, expression is induced by several pro-oxidant conditions (Foletta et al., 1998), including different types of irradiation (Weichselbaum, 1994). However, the activity of AP-1 can also be regulated post-translationally. For example, AP-1 binding to DNA increases upon reduction of critical cysteine residues in AP-1, whereas when the residues are oxidized, its binding activity to the DNA decreases (Abate, 1990). AP-1 binding sites are present in the promoter regions of a large number of genes including collagenase, matrix metalloproteases (MMP), cytokines, growth factors, adhesion molecules, cell cycle

enzymes, receptor kinases, all of which are related to disease processes (Schenk, 1994 ; Sen, 1998).

NF-κB is regulated by redox mechanisms quite differently from AP-1, although ROS can trigger both of these transcription factors. Activation of NF-κB occurs in the cytosol by degradation of the inhibitory protein I-κB, a degradation product that is induced by oxidative conditions and prevented by antioxidant compounds (Nakamura, 1997). Targets for NF-κB include the genes corresponding to cytokines such as interleukins (IL-1, IL-6, IL-8 and TNFα), inducible nitric oxide synthase (iNOS), adhesion molecules, matrix metalloproteineases (MMP), growth factors, major histocompatablity antigens (MHC class 1 and 2) and different acute phase proteins (Baeuerle, 1994). NF-κB activation has been extensively investigated in various human disease conditions and several investigators have reported increased NF-κB activity in human disease conditions.

ROS as a cause of oxidative tissue damage

As described earlier, ROS production is necessary for host defence against invading microorganisms. Homeostasis is always maintained between production of oxidants and the levels of antioxidants. However in certain circumstances, the production of oxidants, over powers the antioxidant, defence system resulting in oxidative tissue damage. The targets for ROS damage include all major groups of biomolecules, summarized as below.

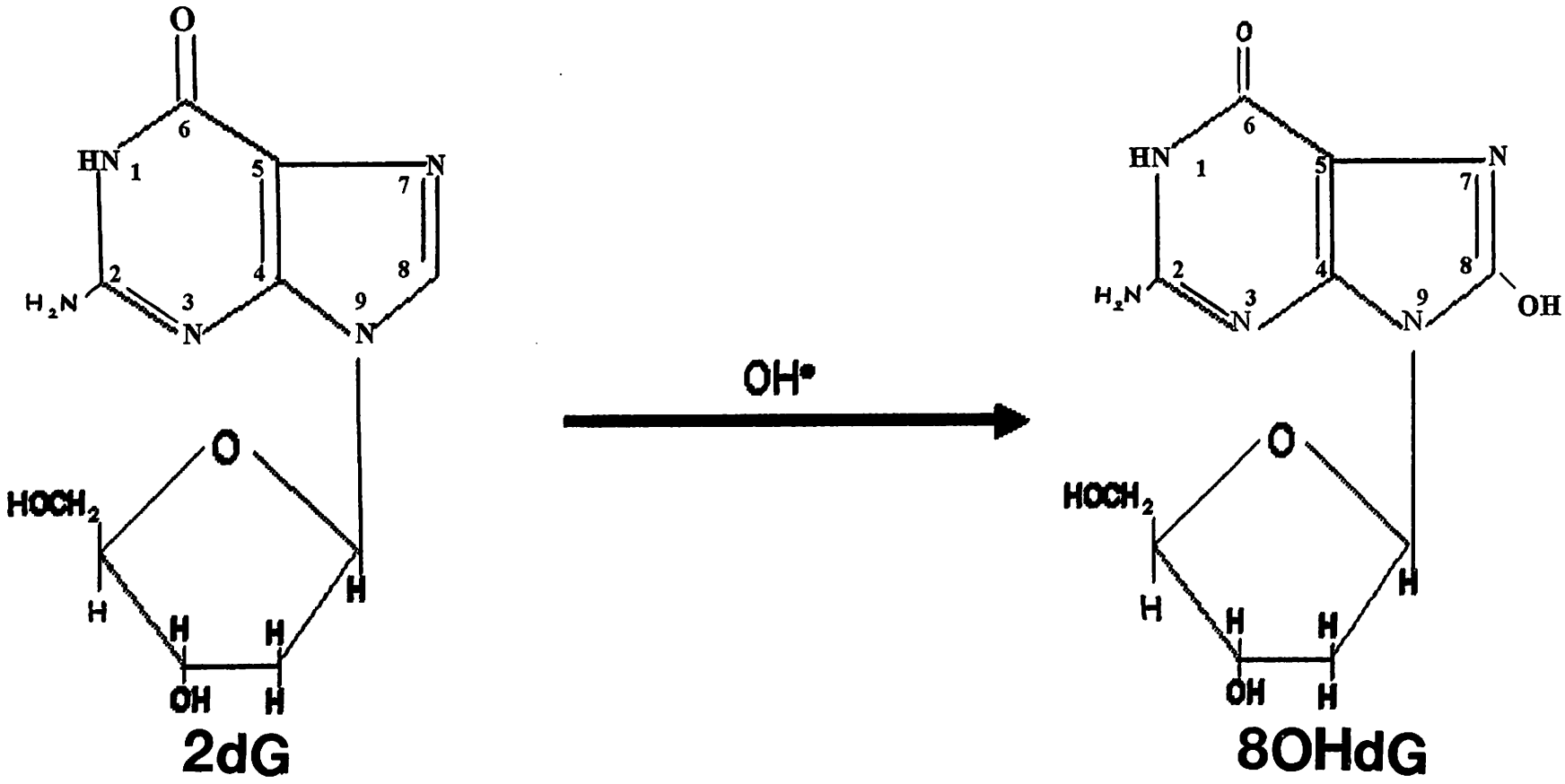


Figure 7: Formation of 8-hydroxy-2'deoxyguanosine

DNA

ROS/RNS have been shown to introduce number of alterations in the DNA e.g. cleavage of DNA, DNA-protein cross links, oxidation of purines, pyrimidines, sugar back bone etc, resulting in structural and functional damage to DNA. Nature has provided the cells with repair systems, which recognize the damaged bases and prevent it from being transcribed. However, in certain conditions, the repair systems fail to do their function, which results in erroneous base paring during replication. This mechanism may partly explain the high prevalence of cancer in individuals exposed to oxidative stress (Marnett, 2000; 2003). The fact that apoptosis in some cases is mediated by ROS (Kamata, 1999) may in part be due to increased mitochondrial permeability, release of cytochrome C, increased intracellular calcium levels, and other effects (Kroemer, 1998). 8-hydroxy-2'-deoxyguanosine (8-OHdG) was first reported in 1984 as a major form of oxidative DNA damage product by Fenton-type reagents and X-irradiation *in vitro* (Kasai et al., 1984). Subsequently several laboratories have shown the elevated levels of 8-OHdG in various human disease conditions in spectrum of clinical samples (Kasai, 1997). The structure of guanosine and 8-OHdG are given in Fig.7.

The concept of ROS as an important factor in cellular and whole organism aging due to damage to mitochondrial DNA is an interesting theory (Ames at al., 1993). This concept was recently challenged by a study based on extensive gene array analysis, suggesting errors in the mitotic machinery and possibly in arachidonic acid metabolism as being important determinants for the aging process (Ly et al., 2000). However, mounting data

do indicate that ROS contribute to aging and conversely, that antioxidant consumption might prolong the life span (Melov et al., 2000).

Lipids

Lipid peroxidation is probably the most explored area of research when it comes to ROS (Herttuala, 1999 ; Halliwell, 1993). Polyunsaturated fatty acids (PUFA), because of multiple double bonds are excellent targets for free radical attack. Cellular membrane lipids are composed of PUFA; therefore oxidation of PUFA results in cell death. The mechanism of atherosclerotic plaques also involves oxidation of low density lipoproteins (LDL), uptake of these oxidized low density lipoproteins (LDL) molecules by phagocytes in the subendothelial space via their scavenging receptor and finally accumulation of these phagocytic cells in the subendothelial space, where they stimulate formation of atherosclerotic plaques (Steinberg, 1997). Accumulation of lipid peroxide has been reported in several other human diseases (Halliwell, 1993). Therefore determining the lipid peroxide content serves as a valuable marker to assess the oxidative damage to lipidsand the antioxidant capacity (Halliwell, 1993).

Proteins

ROS have been shown to react with several amino acid residues, generating anything from modified and more active enzymes to denatured nonfunctioning proteins (Butterfield et al., 1998; Stadtman et al., 1998). Among the most susceptible amino acids are sulfur containing amino acids and aromatic amino acids. General antioxidant enzymes such as SOD, GPx or glutathione reductase (GR) or specific enzymes such as methionine

sulfoxide reductases all these uphold the protection to proteins from oxidative modifications (Lowther et al., 2000).

Antioxidant defense system

The classification of antioxidant defence system is described in Table 5. The important antioxidant molecules and their mode of action are presented in the following section.

Superoxide dismutases (SOD)

SOD was the first genuine ROS-metabolizing enzyme discovered (McCord and Firdovich, 1969). In eukaryotic cells $O_2^{\bullet-}$ can be metabolized to hydrogen peroxide by two metal-containing SOD isoenzymes, an 80-kDa tetrameric Mn-SOD present in mitochondria, and the cytosolic 32kDa dimeric Cu/Zn-SOD. The following is the reaction catalyzed by SOD.

$$2\ O_2^{\bullet-} + 2H^+ \longrightarrow H_2O_2 + O_2$$

In mitochondria, $O_2^{\bullet-}$ is formed in relatively high concentrations due to the leakage of electrons from the respiratory chain. The strictly mitochondrial Mn-SOD (Weisiger and Firdovidh, 1973) is obviously essential to remove $O_2^{\bullet-}$. Lethal neurodegenerative disease – amyotrophic lateral sclerosis (ALS) has been associated with the mutation in the gene for cytosolic SOD (Rosen et al., 1993). Diminished SOD activity results in accumulation of $O_2^{\bullet-}$ which gets converted to hydrogen peroxide and ultimately $OH^{\bullet}$ radical is produced via Fenton reaction.

Table 5. Classification of antioxidant defence system

Endogenous Antioxidants			Nutritional Antioxidants			
Enzymatic	**Non enzymatic**	**Metal – ion-sequestering proteins**	**Vitamins**	**Minerals**	**Carotenoids**	**Polyphenols**
SOD	GSH	Haptoglobin	A, E & C	Zn & Se	Lycopens	Catechin
Catalase	Thioredoxin	Haemopexin			α and β carotene Lutein	Caffeic acid Genistin
Glutathione peroxidase	Lipoic acid	Transferrin			Zeaxanthin	Quercetin
Glutathione Reductase		Ceruloplasmin				
		Metallothioneins				

Catalases

Catalases of many organisms are mainly heme-containing enzymes (Aebi, 1974). The predominant subcellular localization in mammalian cells is in peroxisomes, where catalase catalyzes the dismutation of hydrogen peroxide to water and molecular oxygen. Catalase also has the function in detoxifying different substrates e.g., phenols and alcohols via coupled reduction of hydrogen peroxide. One antioxidative role of catalase is to lower the risk of hydroxyl radical formation from hydrogen peroxide via Fenton reaction (Firdovich, 1999).

Glutathione peroxidases (GPx)

There are at least four different GPx in mammals (GPx1-4) all of them contain selenocysteine (Ursini et al., 1995). GPx1and 4 are cytosolic enzymes abundant in most tissues (Haan et al., 1998). GPx 2 is expressed in gastroinstestinal tissue, whereas GPx3 is found in plasma. All GPx catalyzes the reduction of hydrogen peroxide using GSH as substrate. They can also reduce other peroxides (e.g., lipid peroxides in cell membranes) to alcohols.

$$ROOH + 2GSH \longrightarrow ROH + GSSG + H_2O$$

The catalytic mechanism proposed for reduction of hydroperoxides by GPx involves oxidation of the active site selenolate to seleneic acid. Upon addition of one molecule of GSH, the selenic acid is transformed to a selenylsulfide adduct with GHS, which can be regenerated to the active selenolate and glutathione disulfide by addition of a second molecule of GSH. Thus in the reaction two molecules of GSH are oxidized to GSSG which is subsequently reduced by glutathione reductase (GR). Some data has indicated

that GPx should be of high antioxidant importance under physiological conditions (Jones et al., 1981), while others place the enzymes as important only at events of oxidative stress (Kelner et al., 1990).

Ho et al., (1997) has reported that knockout mice lacking GPx1 developed quite normally and even coped with exposure to oxidative stress such as. Therefore the function of GPx isoenzymes in antioxidant defense is still unclear, but the kinetic properties and widespread distribution still imply that they constitute major contributions to the total protection against oxidative stress. The role of GPx system in detoxification of ROS is represented schematically in Fig.8.

Other GSH related systems

GSH is the most abundant intracellular thiol-based antioxidant, prevalent in millimolar concentrations in all living aerobic cells. Its function is mainly as a sulfhydryl buffer, but GSH also serves to detoxify compounds either via conjugation reactions catalyzed by GSH transferases or directly, as it is the case with hydrogen peroxide in the GPx catalyzed reaction. Oxidized GSH is reduced by the NADPH-dependent flavoenzyme glutathione GR. Another class of proteins intimately related to GSH is the glutaredoxins (Grx), with functions overlapping those of thioredoxins (Trx). A major difference between Grx and Trx, is that, Grx can be reduced by GSH and is capable of reducing GSH mixed protein disulfides formed at oxidative stress, which should play an important role in the total cellular antioxidant defense (Holmgren, 2000).

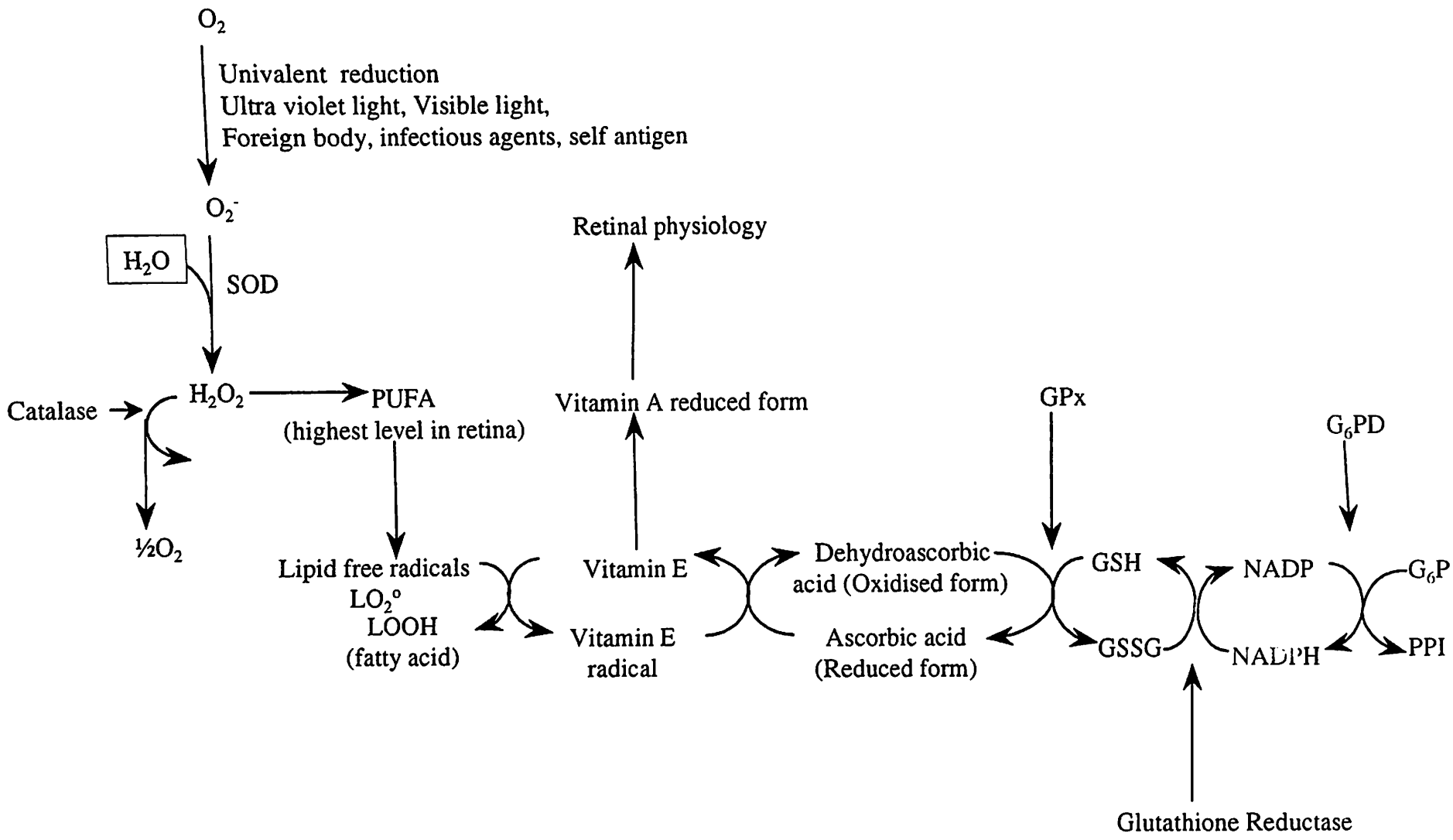


Figure 8: Schematic representation of antioxidant vitamins and GSH system in detoxification of ROS.

Antioxidant defence: sequestration of metal ions

Iron and copper are essential in the human body for the synthesis of variety of enzymes and other proteins that are involved in respiration, redox reactions and other metabolic pathways. Yet these two metals are potentially dangerous by their ability to participate in one-electron transfer reactions. This enables them to be powerful catalysts of autoxidation reactions. For instance, conversion of H_2O_2 to $OH^{\bullet}$ and decomposition of lipid peroxides to reactive peroxyl and alkoxyl radical. It is not only free metal ions that are catalytic. Haem and certain haem proteins can decompose lipid peroxides and interact with H_2O_2 to cause extensive structural and functional damage to the biomolecules. Therefore it is necessary to regulate the metabolism of these metal ions carefully, which are vital for physiological functions but are dangerous when their level exceeds the critical limit. The proteins which are involved directly or indirectly involved in regulation of iron metabolism are transferrin, haptoglobin (Hp) and Haemopexion (Hpx). Ceruloplasmin sequesters Cu^{2+} ions and excretes them in to bile. Metallothioneins can also sequester Cu^{2+} ions and prevent the $OH^{\bullet}$ induced tissue damage (Halliwell and Guttridge, 1999).

Transferrin

Transferrin is a glycoprotein (~ molecular mass 79 kDa) is synthesized by liver and Tf binds Fe^{3+} released by the destruction of RBC. However, Tf does not bind haem and protect against ROS generation by free haem. Tf belongs to the family of APR proteins (Halliwell and Guttridge, 1999).

Haptoglobin

Hp is a glycoprotein is synthesized by liver and it is one of the abundant proteins in the circulation (1.2 – 3 g /L). There are three isoforms of Hp. Hp 1-1 has ~ mol wt of 86 kDa; Hp 2-1 ~ 86-300 kDa and Hp 2-2 ~ 170- 900 kDa respectively. It binds Hb released by intra-vascular hemolysis of erythrocytes as a result of routine damage to these cells as they circulate and also during tissue damage, inflammation and necrosis. As a result of this binding, hemoglobin does not release haem and therefore haptoglobin acts an antioxidant. Like Tf, Hp is also a member of APR proteins (Wuyts et al., 2000).

Haemopexin

Hpx is a 60-kDa glycoprotein. It also belongs to the family of APR proteins. It is synthesized by hepatic parenchymal cells. Since haem (iron-protoporphyrin IX) is a reactive lipophilic molecule of limited water solubility, Hpx maintains haem in a soluble state in aqueous environment (Smith, 1999). The major sources of extracellular haem are Hb from ruptured erythrocytes, myoglobin and enzymes with haem prosthetic groups including peroxides and cytochromes from damaged tissues or secreted myeloperoxidase from either monocytes or polymorphs (Smith, 1999). The iron in haem ring of Hb and myoglobin is in the Fe^{2+} state and essentially remains so when O_2 binds. However, some delocalization of these electrons takes place resulting in the intermediate structure.

$$\textbf{Haem-Fe}^{2+}\textbf{-O}_2 \leftrightarrow \textbf{haem-Fe}^{2+}\textbf{-O}_2^{\bullet-}$$

The $O_2^{\bullet-}$ thus produced can react with H_2O_2 in the presence of iron or copper to produce more powerful oxidant $OH^{\bullet}$. Hpx has been shown to bind haem with high affinity and mitigate haem induced lipid peroxidation (Grinberg et al., 1999).

Ceruloplasmin

Copper ions are powerful catalysts of free radical damage: they convert H_2O_2 to $OH^{\bullet}$, decompose lipid peroxides, catalyze autoxidation reactions (especially of ascorbate) and are highly effective in causing oxidative DNA damage and stimulating peroxidation of low density lipoproteins (Halliwell and Guttridge 1999). Like iron, copper must be handelled carefully. Caeruloplasmin has molecular weight of about 132 kDa. Ceruloplasmin is secreted in to circulation to be excreted in to the bile. Caeruloplasmin is mostly synthesized in liver but lungs and brain has also been shown to synthesize caeruloplasmin (Yang and Young, 1996). Caeruloplasmin has been shown to possess ferroxidase activity; it oxidizes Fe^{2+} to Fe^{3+} and thus facilitates iron loading on to Tf. Thus caeruloplasmin acts an antioxidant by mitigating the generation of radical production by sequestering copper ions and through its ferroxidase activity it also aids in regulation of iron levels (Harris, 1995).

Metallothioneins

Metallothioneins (MT) are proteins with low molecular weight (6.5 – 7.5 kDa) and are found to be synthesized by variety of cell types. MT are rich in sulfur (25-33% if the amino acids are cysteine). They therefore represent a significant portion of total cell protein thiol. Each MT molecule can bind 5-7 ions of metal ions such as Zinc (Zn^{2+}),

silver (Ag^{+}), copper (Cu^{+}), cadmium (Cd^{2+}) and mercury (Hg^{2+}) (Dunn, 1987). Binding of metals to metallothioneins are achieved by association of cysteine-SH groups with metal ion; for example, Cd^{2+} and Zn^{2+} are linked to four cysteine thiolate ligands ($Cys\text{-}S^{-}$) in a tetrahedral arrangement. MT expression is triggered upon heavy metal toxicity or during inflammatory conditions. It has been recently suggested that metallothioneins might exert antioxidant properties. Sequestration of copper ions will diminish radical generation promoted by this metal, Zn^{2+} released from Zn-MT might inhibit lipid peroxidation and high content of –SH groups in MT makes them excellent scavengers of $O_2^{\bullet-}$ and $OH^{\bullet}$. Over expression of MT in cells have been shown to confer protection by H_2O_2 induced oxidative damage; these results suggest the antioxidant activity of MT (Chubatsu and Meneghini, 1993).

Synthetic antioxidants

Desferrioxamine acts as powerful iron chelator and it has been used in treating iron overload diseases (Kontoghiorghes and Weinberg, 1995). Subsequently desferrioxamine has been shown to inhibit Fenton reaction and prevent the formation $OH^{\bullet}$. Desferrioxamine has also been shown to directly quench $OH^{\bullet}$. Thus by preventing the formation desferrioxamine has been shown to inhibit lipid peroxidation. In addition to the above, animals challenged with either oxidative stress or induced inflammation, desferrioxamine treatment has been shown to ameliorate the complications of oxidative stress and mitigate the severity of inflammation (Halliwell and Guttridge 1999).

N –acetlycysteine was developed as an analogue to GSH and it acts an antioxidant by acting as electron sink like GSH (Cotgreaue, 1996). N – acetlycysteine administration has been shown to elicit protective action against oxidative stress in experimental animals and in certain human diseases (Cotgreaue, 1996). Ebselen is a seleno-orgainc compound, that was originally described as GPx mimic (Müller et al 1984). Recently ebselen has been shown to have anti-inflammatory action by its ability to inhibit iNOS expression in experimental uveitis model (Morell, et al 2002).

Biological aspects of RNS

The intensive research surrounding nitric oxide (NO) and its metabolites has given rise to new term the 'reactive nitrogen species' (RNS). By strict chemical criteria RNS encompasses a diverse range of compounds with contrasting and distinct properties, that their only unifying characteristics can be derived from NO. NO is synthesized enzymatically from L-arginine by nitric oxide synthase (NOS) (Bret, 1999). There are three isoforms of NOS, i.e. neuronal NOS (nNOS - NOS1), inducible NOS (iNOS - NOS2) and endothelial NOS (eNOS - NOS3) (Andrew, 1999). NO represents an odd member of free radical family and is similar to $O_2^{\bullet-}$ in several aspects in that, it does not readily react with biomolecules inspite of having an unpaired electron. On the other hand it freely reacts with free radicals, generating mainly less reactive molecules, thus under circumstances NO can act as free radical scavenger.

Formation of RNS in biological systems

In biological systems the primary source of RNS is NO. Through the rapid reaction of NO and $O_2^{\bullet-}$, peroxynitrite ($ONOO^-$) is formed which can result in oxidation, nitrosation (addition of NO) or nitration (addition of NO_2). NO is formed from the enzymatic reaction of NOS, which under certain circumstances can also produce $O_2^{\bullet-}$. The potential sources of $O_2^{\bullet-}$ production in the cell include xanthine oxidase (XOD), and NADPH oxidase (Jarasch et al., 1986). The oxidation reactions (e.g. Oxidation of GSH) occur at the highest chemical yield, but the nitration (formation of nitrotyrosine) or nitrosation (e.g. formation of S-nitrosoglutathione) are also biologically significant since they can alter cell signalling (Wink, 1998). In physiological concentrations NO functions mainly as an intracellular messenger stimulating guanylate cyclase and protein kinases, thereby relaxing smooth muscle in blood vessels and it is called as "Endothelium Derived relaxing Factor (EDRF)". NO has also got the ability to cross the cell membranes and can thereby also transmit signals to other cells, by its ability to modulate gene expression (Moncada and Higgs, 1991).

Like ROS, RNS can also cause damage DNA, lipids and proteins. RNS can act as an oxidizing agent as well as nitrating agent (Stamler, 1994). Cells that are recruited to the site of injury and initiate inflammatory events such as macrophages and activated neutrophils produce relatively high concentrations of NO via iNOS. It has been suggested that this leads to an increase in the formation of $ONOO^-$, which is then used as a part of arsenal against the invading pathogen (Nathan and Hibbs, 1991). Although this is an

important host defence system, similar reactions, as evidenced by the detection of nitrotyrosine (3NTYR), appear to occur during the development of various inflammatory diseases and this has lead to the hypothesis that $ONOO^-$ is an important mediator in such pathologies (Beckman and Koppenol, 1996).

Biomarkers for oxidative stress

Establishing the involvement of free radicals in the pathogenesis of a disease however, is extremely difficult, due to the short lifespan of these species, but also due to lack of sufficiently sensitive technology to detect radicals directly in biological systems (Pyror, 1986). As a consequence of these analytical problems related to oxidant stress and free radical mechanisms of injury, much of the evidence is circumstantial. Therefore, in many diseases it is still not clear whether free radicals are the sole cause of the injury or are formed as the result of the disease (Jaeschke, 1995).

For this purpose, there is a great need for biomarkers of radical damage, which can be used to monitor the involvement of such damage in the pathogenesis of diseases. The term 'biomarker' has been adapted from molecular epidemiology by free radical biologists to describe a molecular change in a biological molecule that has arisen from attack from reactive oxygen, nitrogen or halide species (Offord et al., 2000). It is applied equally to products derived from lipids, DNA, protein and antioxidant consumption, where the chemical nature of the reaction may be protein abstraction, electron transfer or direct addition. Information regarding the nature of the denaturing radical, as well as the localization of the oxidative stress, may be gleamed from the analysis of discrete

biomarkers isolated from tissues/organelles/fluids. Biomarkers have, therefore been used to evaluate the efficacy of many antioxidants *in vitro* and *in vivo*.

Biomarkers may yield information on the three progressive levels to disease outcome: (i) as measurable endpoints of damage to proteins/amino acids, oxidized lipids, DNA bases, (ii) as functional markers of, for example blood flow, platelet aggregation, or cognitive function and (iii) as end points related to specific disease (e.g. lens opacity in cataract). While the clinical symptoms of a disease are endpoints in themselves, they are not suitable in many cases for early detection and therefore, prevention of disease. A series of biomarkers would be preferred, each validated in sequence. To this end, the association between a biomarker and a disease should be defined. A valid biomarker should be

a) A major product of oxidative modification that may be implicated directly in the development of disease.
b) A stable product, not susceptible to artifactual induction or loss during storage
c) Representative of the balance between oxidative damage generation and clearance (i.e the steady state, but also possibly applicable to the measurement of cumulative damage).
d) Determined by an assay that is specific, sensitive, reproducible and robust.
e) Accessible in a target tissue or a valid surrogate sample such as leukocyte and
f) Measurable within the limits of detection of a reliable analytical procedure.

Biomarkers of Protein Oxidation

The biomarkers of protein oxidation include a) carbonyl groups formation b) total protein thiol content c) oxidized aliphatic amino acid residues d) oxidized tryptophan and tyrosine residues, nitrated tyrosine residues. The biomarkers of protein damage are listed in Table 6. In the present study, 3 nitrotyrosine (3NTYR) and carbonyl groups were used as biomarker for protein oxidation. The rational for selecting these markers are discussed in the chapters 2 and 3.

Lipid peroxidation Biomarkers

Biomarkers of lipid peroxidation include a) malondialdehyde (MDA) b) conjugated dienes c) lipofuscin d) lipid peroxides and e) isoprostanes. In the present study thiobarbituric acid reactive substances (TBARS) determination was used as biomarker in samples obtained from patients with ED.

Biomarkers of DNA oxidation

Free radical attack upon DNA generates a range of DNA lesions, including strand breaks and modified bases. $OH^{\bullet}$ attack on DNA leads to a large number of pyrimidine and purine derived base changes. Some of these modified DNA bases have considerable potential to damage the integrity of the genome (Cheng et al., 1992). 8-OHdG is considered to be the early biomarker to assess the intensity of free radical mediated tissue damage during inflammatory conditions (Dizdaroglu, 1991).

Table 6: Biomarkers for assessing oxidative protein damage.

SL. No.	Substrate and oxidative stress	Product
1.	Tyrosine and $OH^{\bullet}$	DOPA
2.	Tyrosine + HOCl	3-Chlorotyrosine
3.	Tyrosine + RNS	3-NTYR
4.	Valine + $OH^{\bullet}$	3- Hydroxy valine
5.	Lysine + OH	3- Hydroxy lysine
6.	Peptide back bone + ROS	Carbonyl group

MATERIALS & METHODS

Materials and Methods

Chemicals

All the fine chemicals used in this study were obtained from Sigma chemical company (St Louis, MO, U.S.A) unless specified. All other reagents used were of the highest analytical grade.

Selection of patients and control subjects

Eales' disease was diagnosed after detailed fundus examination by an ophthalmologist. Other retinal vasculitis conditions, which mimic ED were eliminated by ordering laboratory tests, as described previously in Table 3 (Introduction section). ED patients were not diabetic, non-obese, non-smoking and were non-alcoholic. Prior to phlebotomy, patients were either not taking any antioxidant vitamin supplements or were put on steroid treatment. Active vasculitis (AV) in ED is usually characterized by venous dilatation in the periphery with tortuosity and discontinuity of veins. Perivascular exudates are seen along the peripheral veins and vascular sheathing. In addition to the above, there would be peripheral venous occlusion, retinal edema, neovascularization and vitreous hemorrhage.

Healed Vasculitis (HV) is characterized by the absence of periphlebitis, sclerosis of veins, fibrovascular scar formation and associated vitreous hemorrhage. Patients in HV stage will be followed up and surgical intervention was made depending upon the clinical symptoms and nature of retinal lesions. Healthy volunteers were included to have them as control. They were not diabetic, non-alcoholic and free from systemic diseases. They

were not taking any antioxidant supplements. Physician obtained detailed clinical history from the patients and control subjects. Written consent was obtained from all the participants included in the studies. Vision Research Foundation - Research and Ethics Committee approved all the studies. All procedures pertaining to human subjects strictly adhered to tenets of Helsinki declarations. All the assays in ED and healthy control subjects' samples were run in parallel. To ensure that findings were consistent and independent of experimental variations, intra assay variation was determined and the values are mentioned wherever appropriate.

Separation of monocytes (MC)

Ten ml of venous heparinized blood sample was used for separation of MC from the study subjects. The blood was diluted with equal volumes of PBS (pH 7.4, calcium and magnesium free , Hi Media Laboratories, Mumbai, India) and carefully overlaid on equal volume of ficoll- hypaque density gradient solution (Amersham Biosciences, UK) and centrifuged at 3000 rpm for 30 min at room temperature (RT). After centrifugation, the interface containing mononuclear cells (lymphocytes and monocytes) were carefully aspirated and washed thrice with PBS pH 7.4 supplemented with antibiotic supplements (Hi Media Laboratories, Mumbai, India). After final step of washing, the pellets were carefully mixed with RPMI 1640 medium and were added to 35 mm-diameter tissue culture dishes. The cells were allowed to adhere for 2 hrs at 37°C in a 5 % CO_2 environment. The adherent cell population contained at least 90-95 monocytes by microscopic examination after staining with Wright-Giemsa stain. Other cells appeared to be lymphocytes. More than 95 % of the cells were viable as determined by trypan blue

exclusion test. MC separated in this manner was immediately processed for the investigations. 2.0 X 10^6 cells/ml were used for each investigation unless specified.

Determination of Hydroxyl Radical Generation by MC using Electron Spin Resonance Spectrometer (ESR)

To assess the ability of MC to generate hydroxyl radical *in vitro,* 2.0 X 10^6 cells / ml from respective study subjects were incubated in 0.5 ml of Hank's balanced salt solution (HBSS, Hi Media Laboratories, India) containing 4- pyridyl-1-oxide N *tert* butyl nitrone [4- POBN (10 mM)], ethanol (170 mM), 4- α- phorbol – 12 myristate 13-acetate [PMA (100 ng / ml)] for 15 min at 37° C. After the reaction, the contents were transferred to quartz ESR flat cell cuvette and placed in the cavity of a x - band ESR spectrometer (Varian E 112, Palo Alto, CA, U.S.A) and the spectra was recorded at 25° C. Instrument settings were as follows: microwave power, 10 milliwatts; modulation frequency, 100 kHz; modulation amplitude, 4.0 G; scan time 4 min; and response time 0.5 sec. The receiver gain was set at 6.3 X 10^4.

Determination of SOD activity in erythrocytes

SOD activity was determined by the method described by (Misra and Firdovich, 1972)

Principle

Superoxide is an intermediate formed during the autooxidation of epinephrine to adrenochrome. The ability of SOD to inhibit the autooxidation of epinephrine at pH 9.8 forms the basis for the assay.

Reagents

1. 50 mM carbonate buffer pH 9.8

2. Epinephrine: 1.0 mg was dissolved in 1.0 ml of Milli Q H_2O.

Extraction of Enzyme

1.0 ml of washed RBC was lysed by adding 1.0 ml of water. The contents were mixed well. Then 0.5 ml of hacmolysate was diluted with 3.5 ml of dH_2O. Following this, 1.0 ml of ice-cold ethanol and 600 µL of ice-cold chloroform were added. The contents were mixed well and centrifuged at 5,000 rpm for 15 min and then the supernatant was carefully aspirated and used for the assay.

Procedure

Blank consisted of 1.2 ml dH_2O, Carbonate buffer 1.8 ml. Control tube consisted of 800 µL dH_2O, and 400 µL of epinephrine. The test cuvette consisted of 800 µl of dH_2O, 1.8 ml of carbonate buffer, 50 µl of enzyme extract and 400 µl of epinephrine. The increase in the absorbance was determined in kinetic mode for 4 min a spectrophotometer at 480 nm (Beckman DU 640, U.S.A). Autooxidation of epinephrine to adrenochrome (a pink coloured chromogen) was performed in control tube, without addition of sample (enzyme source). One unit of enzyme activity was defined as the quantity of enzyme required to produce 50 % inhibition in the epinephrine autooxidation. The SOD activity in erythrocytes was expressed as units /g Hb. The intra assay coefficient for erythrocyte SOD was determined to be 15 units / g Hb.

Determination of Hemoglobin

Hemoglobin was estimated by the cyanmethhaemoglobin method of Drabkin and Austin (1932).

Principle

The hemoglobin is treated with a reagent containing potassium ferricyanide, potassium cyanide and potassium dihydrogen phosphate. The ferricyanide forms methhemoglobin, which is converted to cyanmethhemoglobin by the cyanide.

Reagents

1. Ferricyanide-cyanide reagent: This was prepared by dissolving 200 mg potassium ferricyanide, 50 mg potassium cyanide and 140 mg potassium dihydrogen phosphate in a litre of water.
2. Cyanmethemoglobin standard: Purchased from Span Diagnostics Pvt. Ltd., (Surat, India). This was kept in dark at 4°C. It had an equivalent hemoglobin concentration of 60 mg %.

Procedure

20 µl of blood (EDTA anticoagulant) was added to 5.0 ml of the reagent and allowed to stand in RT for 15 min and was read against reagent blank at 540 nm. The standards were diluted in ferricyanide-cyanide solution to obtain a range of concentrations and read in same manner. Hemoglobin values were expressed as g /dl.

Determination of SOD activity in MC

Cells were suspended in HBSS and incubated with 100 ng /ml PMA for 30 min at 37 ° C. After incubation the cells were pelleted by centrifuging at 4,000 rpm for 10 min at 4°C. Then the cells were resuspended in 1.0 ml of lysis buffer containing protease inhibitors such as sodium orthovanadate, phenyl methyl sulphonyl fluoride (PMSF) and aprotinin at the final concentration of 10 µg/ml in PBS pH 7.4. Subsequently lysed by

sonicating (Vitris, U.S.A) 35 K for 30 sec X 2 on ice. Homogenates were clarified to remove the cellular debris by centrifuging at 3,000 rpm for 15 min at 4° C. All protein content determinations were performed by Lowry method using BSA as standard (Lowry et al., 1951). To the 300 µL of the lysate added 100 µL of ice-cold ethanol and 60 µL of chloroform and centrifuged at 5000 rpm for 15 min at 4°C. The rest of the procedure is similar as described above. SOD activity in MC was expressed as units/mg protein. The SOD activity in MC was also performed in the absence of PMA stimulation. The intra assay coefficient for SOD was determined to be 0.5 units / mg protein.

Determination of plasma GSH levels

Plasma GSH was determined by following the method described by Miuo- Lin Hu (1994) with slight modifications. Briefly 0.5 ml of plasma was added to 0.5 ml of ice cold 10 % TCA and kept in ice for 10 min. Then centrifuged at 3000g for 15 min at 4°C. 0.2 ml of the supernatant was taken and mixed with 1.7 ml of 0.1 M sodium phosphate / 5 mM EDTA buffer pH 8.0 and 0.1 ml of O- pthalaldehyde (1 mg / ml in methanol) and again incubated at RT for 15 min. The amount of GSH in the plasma was determined in fluorimeter (Perkin Elmer LS 30, Foster City, CA, U.S.A) by measuring the excitation at 350 nm and emission at 420 nm. Prior to estimation of GSH in clinical samples, the system was calibrated with authentic standard of GSH (0.5 – 6 µM). The amount of GSH in plasma was expressed as µM. The intra assay coefficient for plasma GSH was determined to be 0.28 µM.

Determination of TBARS formation in MC

Cells were stimulated with or without PMA and lysed as described above. TBARS content in the lysates were estimated by following the method described by (Devasagayam and Tarachand, 1987). To 250 µL lysate, 200 µL of ice-cold 10 % TCA was added and mixed well. Then the tubes were allowed to stand in RT for 10 min. After this step the precipitated proteins were pelleted down by centrifuging at 4000 rpm for 15 min. The clear supernatant was added to 1.0 ml of thiobarbituric acid (TBA) solution and heated for 15 min in boiling water bath. The tubes were cooled and the absorbance was measured at 532 nm. TBARS content in MC were expressed as nM MDA / mg protein. The intra assay coefficient for TBARS was found 0.4 nmole MDA/ mg protein.

Determination of Thiobarbituric acid Reactive Substances in erythrocytes (Devasagayam et al., 1987)

Principle

Malondialdehyde (MDA) produced during peroxidation of lipids, served as an index of lipid peroxidation. MDA reacts with Thiobarbituric acid (TBA) to generate a coloured product, which absorbs at 532 nm.

Reagents

1. 10 % trichloroacetic acid (TCA) in dH_2O.
2. TBA : 500 mg of TBA was dissolved in 6.0 ml of 1M NaOH and mixed well, to this added 69 ml of dH_2O.

3. Stock standard MDA: 0.05 ml of 1,1',3,3'-tetraethoxy propanebis (diethyl acetate) [TEP] was made up to 1.0 ml with 0.9 % NaCl and 0.03 ml of 6N HCl and made up to 100 ml with dH_2O.
4. Working standard was prepared by diluting 1.0 ml from stock to 50 ml of dH_2O.

Procedure

The erythrocytes were washed thrice with saline after removal of plasma. To the packed cells 1.5 ml of 10 % TCA was added and was allowed to stand for 15 minutes at RT. The tubes were centrifuged and to the supernatant 1.5 ml of TBA solution was added and heated in boiling water bath for 15 min. After cooling the tubes, the absorbance was measured at 532 nm.

Reagents	**B**	**S1**	**S2**	**S3**	**S4**	**S5**	**T1**
Working Standard (µl)	--	100	200	300	400	400	--
Standard Conc.(nM)	--	5	10	15	20	25	--
Washed Erythrocytes (ml)	--	--	--	--	--	--	1
H_2O (µl)	500	400	300	200	100	--	--
TCA (ml)	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Incubate at RT for 15 minutes.							
TBA (ml)	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Kept in boiling water bath for 30 min, cooled and absorbance read at 532 nm. The values were expressed as nM MDA / g of Hb.							

Determination of Iron Copper and Zinc content in MC

Cells stimulated with or without PMA were washed twice with PBS by centrifuging at 3,000 rpm at 4° C for 15 min. To cells, added 1.0 ml of concentrated nitric acid: perchloric acid mixture (5:1) and heated in China dish in fume hood for 30 min. The ash formed was finally dissolved in 2.0 ml of milli Q water. The levels of iron , zinc and copper were estimated by Atomic Absorption Spectrophotometer (Perkin Elmer 2380, CA, U.S.A) as described earlier (Hinks et al., 1982). Standard graph was generated using authentic atomic absorption standard solutions for iron copper and zinc (Sigma chemical company, U.S.A). The content of trace elements were expressed as µg / mg protein. Intra assay coefficient for iron, zinc and copper were found to be 0.05, 0.06 and 0.03 µg / mg protein respectively.

Measurement of MC Viability In Vitro

Separated MC (10^6/ml) from patients with ED and healthy control subjects were incubated with the individual autologous serum (20 % vol /vol diluted with HBSS) for 1 hr at 37°C. In the second set of experiments, cross incubations were carried with heterologous sera. MC was counted before and after 1 hr of incubation. Cell viability was determined by trypan blue (0.1 % wt / vol) exclusion technique. The cells were counted in Neubauer counting chamber. Viability rate was expressed as the ratio of cell counts before and after the incubation with sera as the (%).

Western blot analysis of iNOS expression in MC

Cells were suspended in PBS pH 7.4, calcium and magnesium free (Hi Media Laboratories, India) containing protease inhibitors, sodium orthovanadate, phenyl methyl sulphonyl fluoride (PMSF) and aprotinin (each 10 µg / ml). The cells were then lysed using a sonicator (Vitris Inc., NY, USA) 15K for 15 sec X 2 on ice. Then the lysates were clarified to remove cellular debris by centrifuging at 4,000 rpm for 10 min at 4°C. The protein content in the supernatant was determined by Lowry method (1951) using BSA as the standard. 50 µg of protein was loaded on to 9 % SDS PAGE (Bio Rad, Richmond, CA, U.S.A).

After electrophoresis, the proteins were transferred on to PVDF membranes (Amersham Biosciences, UK) using Bio Rad Trans blot equipment. Transfer was done using transfer buffer consisting of 3.3 g of Tris base, 14.4 g glycine and 1.0 g SDS dissolved in 800 ml of milli Q water and the final volume was made up to 1L with 200 ml methanol, pH 8.2-8.4. Transfer was performed for an hr at 100 V (400 mA). After the complete transfer, the membrane was then blocked with 5 % skimmed milk powder in PBS pH 7.4 for an hour at RT. After thorough washing of the membrane with PBS, it was subsequently incubated with anti human iNOS antibody (1:1000, rabbit polyclonal, Santa Cruz biotechnology, CA, U.S.A) over night at 4°C in rocking platform. Following this step, the membrane was again washed with PBS and probed with secondary antibody anti rabbit IgG HRP conjugate (1:2500, mouse monoclonal, Sigma Chemical Co., MO, U.S.A) for 2 hrs at RT. Then iNOS protein was visualized using diamino benzidine/cobalt chloride solution (Sigma Chemical company, U.S.A). The amount of iNOS protein was quantified using an

image documentation system (Image Master, Visual Documentation System, Amersham Biosciences, UK) and the values are expressed as pixel intensity / 50 µg of protein.

Determination of 3NTYR in MC

For quantification of 3NTYR by RP HPLC, Millipore water with 18 MΩconductance (Millipore Co, Bedford, MA, U.S.A) was used. The water was further treated with 10 µM diethylenetriaminepentaacetic acid (DTPA) to remove iron, which is known to produce artifacts during protein hydrolysis and interfere in subsequent steps. In addition to this, the water treated in this way was further filtered through 0.22 µm filter (Millipore Co, Bedford, MA, USA), autoclaved and used for the experiments. 3NTYR were determined by following the method described by Crow and Ischiropoulos (1996) with slight modifications.

In brief, the proteins from cell lysates were resuspended in 6N HCl and hydrolyzed by heating at 110° C for 16h. Hydrolysates were ultrafiltered with 10 kDa cut off filters (Millipore Co, Bedford, MA, U.S.A). The acid was removed by lyophylization. The lyophylized powder was dissolved in 1.0 ml of treated milli Q water and stored at – 80° C until further analysis. For determination of tyrosine, 10 µl aliquot of the sample from the above preparation was derivatized with 10 µl of (pre column) with orthopthaldehyde (OPA, 15.0 mg / 500 µl 0.4 M borate buffer pH 10.2 containing 10 µl of 3 mercaptopropionic acid and 50 µl of borate buffer. 50 µl was injected in to the exchange column (Amino Quant 100 A°, 5 µm, 2.1 X 200mm, Agilent Technologies, U.S.A). The analysis were done using the Agilent 1100 series RP HPLC system equipped with a

thermostat column compartment, binary pump and variable wavelength UV detector. Prior to estimation of amino acid content (tyrosine) in protein hydrolysates, the system was calibrated with mixture of authentic amino acid standard mixtures in the range of 10 pM – 1.0 nM (Agilent Technologies, U.S.A).

The chromatographic conditions for amino acid analysis were as follows: Mobile phase A consisted of 20mM sodium acetate containing 0.018 % triethyl amine and 0.15 % tetrahydrofuran pH adjusted to 7.2 with 2 % acetic acid. Mobile phase B = 20% of 100 mM sodium containing 40 % acetonitrile and 40 % methanol (pH was adjusted to 7.2 with 2 % acetic acid).

Gradient program used were as follows: 0 – 17 min, 0 – 60 % B (0.5ml/min); 17.1 – 24 min, 60 – 100 % B (0.5 – 0.8 ml/min); 24.1 – 26 min, 0 % B (0.5 ml/min) with post run time 5 min. The elution was monitored at 338 nm and the column temperature was maintained at 40° C. Data acquisition was performed using Chemstation Software (Agilent Technologies). The intra assay coefficient for tyrosine was found to be 0.15 µg / mg protein.

For estimation of 3NTYR, the system was first calibrated with authentic standard of 3NTYR (Sigma Chemical Co., U.S.A) in the range of 5 picomole/L – 25 picomole/L. The presence of 3NTYR in the clinical samples was confirmed by spiking with known amount of authentic 3NTYR standard. 50 µl from the aliquot (protein hydrolysate) was directly injected on to RP C18 column (5 µm, 300 A°, 2.1 X 200 mm, Zorbax, U.S.A) and eluted with 100 mM ammonium formate buffer pH 3.5 and acetonitrile. The elution

consisted of 16-min linear gradient consisting of 5-80 % acetonitrile followed by 4 min at 80 % acetonitrile (v/v). The flow rate was kept at 0.5ml/min, with column temperature being maintained at 37°C. Elution was monitored at 365 nm. Under acidic conditions, tyrosine absorbs at 280 nm, while 3NTYR absorb specifically at 365 nm. The amount of 3NTYR was expressed as µg / mg protein and as a ratio of 3 NTYR / Tyr. The intra assay coefficient for detection of 3 NTYR was found to be 0.012 µg / mg protein.

Immunolocalization of iNOS and 3NTYR in ERM of patients with ED

Eight ERM tissues were processed in automated tissue processor (Leica Instruments, Germany) and finally embedded in paraffin wax blocks. 5 µm sections were obtained using microtome (Leica, JUNG RM 2045, Germany) and the paraffin sections were transferred on to poly-L-Lysine coated glass slides. After deparafinization, the sections were incubated with 3.0 % hydrogen peroxide for 10 min to quench the endogenous peroxidase activity. After this step, the sections were gently washed with PBS pH 7.4 (5 min, two changes each) and then exposed to 2 % normal rabbit serum (Dako, Copenhagen, Denmark) for 30 min at 4°C. Sections were again washed with PBS (5 min , two changes each) and then probed with either anti iNOS antibody (rabbit polyclonal, 1: 500, Santa Cruz Biotechnology, CA, U.S.A) or anti 3NTYR antibody (rabbit polyclonal, 1: 750, Cell Signalling Technologies, U.S.A) for 2 hours at RT in humid chamber. Following this, the sections were washed in PBS (5 min, two changes each) and then incubated with biotinylated anti rabbit IgG antibody (1: 1000, Dako) for 45 min which was followed by incubation with avidin-biotin complex (Dako) for 15 min at RT. The

sections were washed gently with PBS (one minute) and developed with 3,3' diaminobenzidine (DAB) substrate kit (Dako).

For histologic correlation, the sections were conterstained with hematoxylin for 45 seconds, and then mounted (Entellan, Merck, Darmstadt, Germany) and coverslipped. The negative controls for immunohistochemistry included omission of the primary antibody and the use of non-immune serum in place of the primary antibody. In this case immunoreactivity for iNOS was abolished. However, in case of nitrotyrosine, primary antibody was preadsorbed with 1.5 mM nitrotyrosine for 30 min before immunostaining. This abolished immunoreactivity for nitrotyrosine. Photographic documentation (ASA 100 film, Eastman Kodak, Rochester, NY, U.S.A) were performed with an optical microscope (Nikon, Japan).

Hematoxylin and Eosin staining

Introduction

If unstained sections of tissue are examined under the microscope with transmitted light, little details other than nuclear and cellular boundaries can be identified. Staining the sections with one or more dyes permits the evaluation of the physical characteristics and relationships of the tissues and their constituent cells. This is facilitated if two contrasting stains are used such as hemotoxylin (which stains the nuclear detail) and eosin (which stains the cytoplasmic details of the cell and extracellular tissues). The most commonly used routine staining method in histopathology is hematoxylin - eosin.

Harri's Hematoxylin

Preparation

Haematoxylin	5.0 g
100% ethyl alcohol	50.0 ml
Potassium or ammonium alum	100.0 g
Distilled water	1000.0 ml
Mercuric oxide	2.5 g

Alum was in water by stirring in a boiling water bath. Simultaneously, hematoxylin was dissolved in alcohol. After completely dissolving alum, the two solutions were mixed together and boiled in direct flame for 2-3 min. Then mercuric chloride was slowly added and then boiled till the color changes to dark purple. Then the solution was cooled by immersing in cold water. Then the stain was filtered and stored in dark colored bottle.

Eosin stock solution

Eosin Y, water-soluble	1.0 g
Distilled water	100.0 ml

Phloxine stock solution

Phloxine B	1.0 g
Distilled water	100.0 ml

Eosin-Phloxine working solution

Eosin stock solution	100.0 ml
Phloxine stock solution	10.0 ml
95% Alcohol	780.0 ml
Glacial acetic acid	4.0 ml

Staining Procedure
The slides were deparaffinized by the following protocol

- Deparaffinize the slides

- Xylene I ..... 15 min
- Xylene II ..... 15 min
- 100 % alcohol (Methanol) ..... 4 min
- 80 % alcohol ..... 4 min
- 60 % alcohol ..... 4 min
- 40 % alcohol ..... 4 min
- Water ..... 4 min

After deparaffinization, the slides were stained in Harri's hematoxylin solution for 8 min and then washed in running tap water for 3 min. Subsequently differentiated in 1 % acid alcohol by dipping the slides in the jar. Then the slides were washed in water and then placed in ammonia water, until the sections are bright blue and again washed in water and then counter stained with eosin-phloxine solution for 2 min and then dehydrated by rinsing through in 95 % alcohol, absolute alcohol and xylene. After this step, the slides were air dried and mounted.

Determination of protein carbonyl content in plasma

Principle

The reactive carbonyl groups formed in the proteins are made to react with 2,4-dinitrophenylhydrazine (DNPH), to form hydrazone. The hydrazones have characteristic absorption at 370 nm, which was measured spectrophotometrically.

Procedure

Carbonyl content was determined by following the method described by Levine et al (1990) with slight modifications in the protocol. In brief, 1.0 ml plasma sample was taken

in duplicates; one tube was marked as test and the other as control. To the test 4.0 ml of 10 mM 2,4-dinitrophenylhydrazine (DNPH) prepared in 2.5 M HCl was added and to the control 4.0 ml of 2.5 M HCl alone was added. The contents in the tubes were mixed thoroughly and incubated in dark (room temperature) for 1 hr. The tubes were mixed intermittently every 15 min. Then 5 ml of 20 % TCA (w/v) was added to both the tubes and left in ice for 10 min. The tubes were then centrifuged at 3,500 rpm for 20 min to obtain the protein pellet. The supernatant was carefully aspirated and discarded. This was followed by second wash with 10 % TCA as described above. Finally the precipitates were washed three times with 4.0 ml of ethanol: ethyl acetate (1:1, v/v) to remove unreacted DNPH and lipid remnants. The final protein pellet was dissolved in 2.0 ml of 6 M guanidine hydrochloride and incubated at 37°C for 10 min. The insoluble materials were removed by centrifugation (3500 rpm for 15 min).

Carbonyl content was determined by taking the spectra of the representative samples at 355-390 nm (Beckman DU 640, Fullerton, CA, U.S.A). Each sample was read against the control sample (treated with 2.5 M HCl). The carbonyl content was calculated from peak absorption (370 nm) using an absorption coefficient (ε) of 22,000 M^{-1} Cm^{-1}. Protein carbonyl content was expressed as nmole/mg protein. The protein content was determined by Lowry method using BSA as standard. The intra assay coefficient for carbonyl content were determined to be 0.12 nmole / mg protein.

Separation of Leukocytes

5.0 ml of heparinized blood was collected from the study subjects. The blood was diluted with equal volumes of PBS (pH 7.4) and carefully overlaid on ficoll hypaque density gradient solution. The tubes were then centrifuged at 3000 rpm for 20 min at RT. After centrifugation, three layers were formed. The top layer (plasma) was carefully aspirated and stored at - 70°C for analysis of glutathione (GSH), the middle layer which contained leukocytes were aseptically aspirated and were washed twice with PBS and then immediately processed for DNA isolation. The bottom layer (erythrocytes) was washed with 0.9 % sodium chloride (V/V) and was used for determining SOD activity and TBARS content.

DNA isolation

DNA isolation from leukocytes for determination of DNA adducts was performed by following the method described by (Helbrock et al.,1999) and as modified by (Cadet et al.,2000). Prior to isolation of DNA, all the reagents and buffers were prepared in autoclaved Milli Q water of 18 MΩ conductance. The reagents and buffers were passed through Chelex 100 resin columns (Bio Rad, CA, U.S.A), to remove trace elements, which can introduce artifacts during determination of DNA adducts.

The purified reagents and buffers were filtered through 0.22 µm sterile filter (Millipore Co, Bedford, MA, U.S.A), degassed to remove dissolved oxygen, purged with nitrogen gas with sterile probe and then used for the experiments. Typically, to the leukocyte pellet 1.5 ml of lysis buffer A [320 mM Sucrose, 5mM $MgCl_2$, 10 mM Tris-HCl, 100

µM desferrioxamine and 1.0 % triton X 100 (V/V)] was added and gently mixed by vortexing and then nuclei were collected by centrifugation at 1500g for 10 min at 4°C. The nuclear pellet was washed with 2.0 ml of buffer A and nuclei are obtained after centrifugation (1500g) for 10 min at 4°C. To the nuclear pellet, 600µl of buffer B [10mM Tris-HCl, 5mM EDTA-Na_2, 150 µM desferrioxamine pH 8.0] and 30 µl of sodium docecyl sulfate (SDS) was added and the resulting suspension was vortexed gently several times to allow the lysis of nuclei. After this step, 30 µl of RNase A (MBI Fermentas, U.S.A) was added and incubated at 50°C for 25 min. Subsequently, 30µl of protease (Quiagen, 20 mg/ml in water) was added and the resulting mixture were incubated at 37°C for an hour and 15 min. The solution was then centrifuged for 15 min at 5000g (4°C) and the supernatant was transferred on to fresh centrifuge tube. To this 1.2 ml of sodium iodide solution [7.6 M NaI, 40mM Tris-HCl, 20 mM EDTA-Na_2, 200µM desferrioxamine pH 8.0) and 2 ml of ice cold iso-propanol were added. Then the tubes were gently inverted several times (by hand) to precipitate out DNA.

DNA was collected by centrifugation (5000 g) for 15 min at 4°C. The DNA was washed again with 40 % iso-propanol (V/V) and then final wash were given with 70 % cold ethanol. The tubes containing DNA in ethanol were left at - 20°C to allow maximum precipitation. After this step, ethanol was evaporated under stream of nitrogen gas using sterile probe. Care was taken not to over dry the pellet as this can introduce artifact. Then DNA was dissolved in sterile water treated as mentioned above. Quantification and purity of DNA in the samples were determined by measuring the [A_{260} nm of 1.0 = 50 µ g

DNA/ml] at A_{260}/A_{280} nm absorbance ratio (Beckman DU 640, Fullerton, CA, U.S.A). All samples had a ratio of 1.75 to 1.85 at A_{260}/A_{280} nm.

Enzymatic Hydrolysis of DNA

5.0 µg of DNA was hydrolyzed by adding 10 units of Nuclease P_1 stabilized in [0.3M sodium acetate, 10 µM $ZnSO_4$) and incubated for one hour at 37°C. Dephosphorylation of the resulting nucleotides was achieved by adding 5 units of alkaline phosphatase stabilized in buffer (200 mM Tris-HCl and 200 µM EDTA-Na_2). The resulting mixture was further incubated at 37°C for 30 min. The mixture was then concentrated on 10 kDa molecular weight cut-of filters (Centricon, Millipore Co., U.S.A), the filtrate was lyophilized and stored at –70°C until further analysis.

Derivatization and analysis by GC/MS

The method described by (Rehman et al., 2000) was used for derivatization and quantification of 8-OHdG. In brief, lyophilized DNA hydrolysates samples were trimethylsilylated with bis (trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% trichlorosilane and acetonitrile (1.5:1 by V/V) at 140°C for 30 min in glass vials sealed with polytetrafluroethylene caps under nitrogen gas. Prior to determination of 8-OHdG in the samples, the system was calibrated with authentic standards of 8-OHdG (0.1 pmole/L to 2.5 nmole/L) and 2dG (10 nmole/L – 30 nmole/L) respectively.

Derivatized samples were analysed using a Shimadzu. QP 5000 GC-MS (Japan) equipped with an auto sampler and computer workstation. The injection port and the

GC/MS interface were kept at 250°C and 290°C respectively. Separations were carried out on a fused silica capillary column HP5-MS - 30 mts X 0.25mm X 0.25 µm (J & W Scientific, CA, U.S.A). Helium was used as the carrier gas with flow rate of 0.6ml / min. Derivatized samples (2 µl) was injected in to GC injection port using split ratio (5:1). Initial column temperature was maintained at 200°C for 3 minutes there after increased to 280°C @ 3° C / min. Selected ion monitoring was performed using electron ionization mode at 70ev with ion source maintained at 190°C. The amount of 8-OHdG in DNA samples were expressed as number of 8-OHdG / 10^6dG. The intra assay coefficient was found to be 0.55 (8-OHdG / 10^6 dG).

Protein content determination by Lowry Method (Lowry et al. 1951)

Principle

In this method proteins are allowed to react with alkaline copper sulphate solution to form copper-peptide bond protein complexes. When Folin-Ciocalteau reagent is added- the copper protein complexes that reduces the phosphomolybidic acid in the reagent to give blue coloured complex, which is read at 660 nm.

Reagents

1. Sodium carbonate: 2.0 % solution in 0.2N NaOH
2. Copper sulphate: 0.5 % solution in 1.0 % sodium potassium tartrate.
3. Alkaline copper solution: 50 ml of reagent 1 was mixed with 1.0 ml of reagent 2.
4. Standard bovine serum albumin (BSA): 1mg / ml in dH_2O.

5. Folin- Ciocalteau reagent: One volume of Folin-Ciocalteau reagent (E-Merck, India) was diluted with one volume of water prior to use.

Procedure

To the protein sample 5.0 ml of alkaline copper sulphate solution was added and allowed to stand at room temperature (RT). Then 1.0 ml of Folin-Ciocalteau reagent was added and allowed to react for 20 min at RT. Then the absorbance was measured at 640 nm. The amount of protein in the test sample was calculated from the standard graph generated using BSA standard (50 – 250 µg). The protein content in the serum was expressed as g/dL, in case of purified protein and in cell lysates it was expressed as mg / ml.

Polyacrylamide Gel Electrophoresis (PAGE) (Davis, 1964).

Reagents

1. Acrylamide (30 %) was prepared by weighing 29.2 g of acrylamide and 0.8 g of N, N'bismethylene acrylamide and dissolving in 50 ml of Milli Q water and then making up the final volume to 100 ml with Milli Q water.
2. 1.5 M Tris-HCl pH 8.8 in dH_2O and stored at 4°C in brown bottle.
3. 0.5 M Tris-HCl pH 6.8 in dH_2O and stored at 4°C in brown bottle.
4. 10 % ammonium persulfate in dH_2O
5. N,N,N',N; - tetraethylenediamine (TEMED) – (Bio Rad, CA, USA).
6. 10 % sodium dodecyl sulfate (SDS) in dH_2O.
7. Sample buffer : dH_2O 4.0 ml
   0.5 M Tris-HCl pH 6.8 1.0 ml
   Glycerol 0.8 ml

10 % SDS	1.6 ml
β mercaptoethanol	0.4 ml
0.05 % bromophenol blue	0.2 ml

For SDS PAGE, one volume of sample was mixed with 3 volumes of sample buffer, boiled the sample in water bath for four min and then loaded on to the gel. For native PAGE (NPAGE) the sample buffer was prepared by omission of 10 % SDS and β mercaptoethanol.

8. Running Buffer : 0.66 g Tris base, 2.88 g glycine and 1.0 g SDS dissolved in 1 liter of water (pH 8.3). For NPAGE the buffer was prepared by omission of SDS.
9. Stain: Coomassie brilliant blue R 250 (0.025 %) was prepared by dissolving125 mg Coomassie brilliant blue R 250 in 25 ml of 70 % perchloric acid and making up the final volume to 1L with dH_2O. The contents were stirred at room temperature for 1 hour and filtered through Whatman No.1 filter paper and used for staining and stored in dark coloured bottle.

Protocol for preparation of separating gel

Reagents	**12 %**	**9 %**	**7.5 %**
Distilled water (ml)	2.5	4.5	4.85
1.5 M Tris pH 8.8 (ml)	2.5	2.5	2.5
10 % SDS (µl)	100	100	100
30 % Acrylamide (ml)	4.0	3.0	2.5
10 % APS (µl)	50	50	50
TEMED (µl)	5	5	5

Protocol for preparation of stacking gel (4 %)

Reagents	Volume
Distilled water	6.1 ml
0.5 M Tris pH 6.8	2.5 ml
10% SDS	100 µl
Acrylamide (30%)	1.3 ml
TEMED	10 µl
APS (10 %)	50 µl

Electrophoresis was performed using Bio Rad - mini Protean III system (CA, U.S.A). After electrophoresis, the gels were stained for 30.0 min in Coomassie brilliant blue R 250, and then destained using 7.0 % acetic acid and methanol mixture and then stored under dH_2O.

Silver staining

Principle

Silver staining was done to detect proteins on NPAGE by the method described by Morrissey (1981). Silver staining is based on binding of silver ions to proteins. After electrophoresis, proteins are fixed, exposed to silver nitrate and developed to form a black precipitate of silver.

Procedure

After electrophoresis, the gels were fixed using 30 % methanol and 10 % acetic acid for 30 min. The gels were then washed in Milli Q water for 30 min with intermittent change of water. After this step the gels were treated with 10 % glutaraldehyde for 30 min and then washed thoroughly in Milli Q water for 30 min with change of water every 10-min. This step was followed by incubation with 0.1 % silver nitrate for 30 min. Then the gels were briefly washed with water for 2-3 min and then developed using 3.0 % sodium carbonate containing 100 µl of 40 % formaldehyde. After the visualization of protein bands, the reaction was arrested by addition of saturated citric acid solution until complete neutralization of sodium carbonate was achieved. The gels were then stored under water.

Protein purification

A new protein was identified by running the serum samples (25 µg / 50 µL) on 7.5% native polyacrylamide gel electrophoresis – (NPAGE). After electrophoresis the gel was stained with coomassie brilliant blue R 250. Samples showing the presence of new protein were stored at - 20°C until they was subjected to purification. Serum samples from six to eight patients with ED in AV stage, was pooled (1.5 g protein/ 25 ml serum) and subjected to 70 % ammonium sulfate precipitation. The precipitate was dialyzed against 10 mM phosphate buffer pH 7.4, for 48 hrs with frequent changes of buffer at every 4 to 6 hrs interval. After dialysis, the sample was centrifuged at 5000 rpm for 30 min. at 4° C, the supernatant was subjected to further purification by preparative gel electrophoresis. During each step of purification, protein content was determined by Lowry method (1951). Preparative electrophoresis (Bio Rad 491, Hercules, CA, U.S.A)

was done on a 5 % NPAGE and the proteins were eluted using 0.05 mM Tris-glycine buffer, pH 8.3. Fractions of 5.0 ml were collected at a flow rate of 1.0 ml/min using continuous buffer elution system (Bio Rad Echno PumpEP1). All the fractions were screened at 280 nm using spectrophotometer (Beckman DU 640, Fullerton, CA, USA) for protein content and to asses the elution profile. They were also subjected to NPAGE and silver staining (Morrissey, 1981) was performed. The fractions positive for the protein were further subjected to anti TBARS activity. The active fractions were pooled and stored at -20°C until further purification. During every stage of purification, the presence of the protein was confirmed by performing 7.5 % NPAGE along with positive serum obtained from patients with Eales' disease.

High Performance Liquid Chromatography (HPLC)- Purification of the protein.

Active fractions from preparative electrophoresis were pooled and protein content was measured and then concentrated by lyophilization using Virtis freeze drier (NY, USA). The protein concentrate (50 mg) was further purified in batches using reversed phase HPLC [LKB Bromma, Sweden].

Solvent A consisted of 0.05 % trifluroacetic acid (TFA) in Milli Q water (Millipore Co, MA, U.S.A). Solvent B consisted of 80 % acetonitrile and 0.05 % TFA in Milli Q water. The separation was performed using linear gradient 0.0 – 20.0 min 0.0 – 100 % B. The fractions were subjected to silver staining and anti TBARS activity. Active fractions were pooled and rechromatographed in the same conditions until the protein resolved as a homogenous peak. The purity was further checked by sodium docecyl sulphate (SDS) PAGE electrophoresis followed by silver staining. The purified protein was dialyzed

against 10 mM N- (2hydroxyethyl) piperazine –N'- (2ethanesulfonic acid) [HEPES] buffer pH 7.4, and used for further characterization studies.

Purification of Protein from Vitreous

Purification of protein from vitreous was done by adopting the same procedure used for serum, but by omission of ammonium sulfate precipitation. The undiluted vitreous samples (2 mg / 4 ml) from six patients with ED were pooled, homogenized with phosphate buffered saline pH 7.4 and centrifuged at 5000 rpm for 30 minutes at 4°C. The protein content in the supernatant was measured by the method of Lowry et al. (1951). The supernatant (3.0 ml) was directly subjected to 5 % preparative polyacrylamide gel electrophoresis. The fractions were analyzed as per serum. The active fractions were pooled and purified further by RP HPLC as described earlier.

Determination of anti TBARS activity of 88 kDa protein in the presence of thiol inhibitors

When the TBARS assay was performed in the presence of 100 ng of the purified 88 kDa protein to 1.0 ml of erythrolysate, MDA levels were decreased. This property was used to identify the presence of 88 kDa protein during its purification in fractions collected from preparative electrophoresis and HPLC. The same assay was carried out to assess the effect of various thiol inhibitors such as parachlromercuric benzoate (PCMB) and 5,5' dithiobis (2-nitrobenzoic acid) DTNB.

Molecular weight determination

This was carried out using a gel permeation HPLC column (TSK 3000, Amersham Pharmacia, UK) and Sigma molecular weight markers, along with these markers, 2 µg of

purified protein was loaded to gel filtration column. The proteins were eluted with 10 mM phosphate buffer pH 7.4 containing 20 % methanol in 50 mM sodium chloride, at a flow rate of 0.2ml/min. The elution was monitored at 280 nm (LKB Bromma UV detector). Molecular weight was calculated from the graph plotted with retention time against log molecular weight. In addition to the above, molecular weight of the purified protein was also determined by SDS PAGE. In both the methods, the molecular weight of the new protein was found to be ~ 88 kDa. Therefore it was refereed as 88 kDa protein.

Determination of Isoelectric point (pI).

Determination of pI of 88kDa protein was performed using Mini-Portean II tube cell apparatus (Bio Rad, U.S.A). In brief, the capillary tubes were filled with monomer solution consisting of 9.2 M urea (5.5 g), 4 % acrylamide (total monomer 1.33 ml), 20 % triton X 100 (2 ml), kaleidoscope pI marker i.e 1.6 % Bio- Lyte 5/7 ampholyte (0.4 ml) and 0.4 % Bio- Lyte 3/10 ampholyte (0.1 ml) (Bio Rad) dH_2O, 0.01 % ammonium sulphate (10 µl) and 0.1 % TEMED (10 µL). This makes total volume of 10 ml sufficient to cast a set of 8 capillaries. The purified 88 kDa protein (5 µg/20µl) was mixed with 25 µl of sample buffer containing, 9.5 M urea, 2.0 % triton X 100, 5 % β mercaptoethanol and 1.6 % 5/7, 3/10 ampholyte markers. Then incubated at room temperature for 15 min. During the incubation time, the gel was pre-electrophoresed (300V/15min) with the upper chamber buffer containing 100 mM NaOH and lower chamber containing 10 mM H_3PO_4. After pre-run the buffers were discarded and the sample was loaded on to the tubes and overlaid with sample buffer 20 µl , which contained 9 M urea along with 0.8 % 5/7

ampholyte and 0.2 % 3/10 ampholyte. Then the focussing was performed by running the gels for 500V for 15 min and then increasing to 750V and made to run for 3.5 hrs. After the completion of the run the pI of the 88 kDa protein was determined by comparing with samples that contained standard proteins with ampholytes and with that of 88 kDa protein.

Thermal denaturation studies

Purified 88 kDa protein (0.5 µg / 50 µl) was heated in various temperatures for different time intervals and anti TBARS activity was determined to assess the thermal stability of the protein.

Determination of total thiol content of 88 kDa protein

Determination of total thiol content was performed by following the method described by Miao Lin Hu, 1994.

Reagents

DTNB 10 mM (4 mg/ml) was prepared in absolute methanol. Tris base (0.25M) – EDTA (20 mM) buffer, pH 8.2.

Procedure

100 µg /ml of 88 kDa protein was mixed with 1.0 ml of Tris – EDTA buffer, followed by addition of 25 µl of DTNB solution and 800 µL of methanol, the tubes were capped and incubated at dark for 20 min. The contents were then spun at 3000 rpm for 10 min and the supernatant were carefully aspirated and absorbance was measured at 412 nm and subtracted form DTNB blank and a blank without DTNB. The thiol content was determined using molar absorption coefficient ε 13,600 $cm^{-1}M^{-1}$.

Periodic acid Schiff staining (PAS) for glycoprotein staining (Gardilone et al., 1998)

Principle

PAS stain is usually used to detect the presence of glycoproteins in tissue sections. Later this method was extended to detect glycoproteins resolved in PAGE. Periodic acid brings about oxidative cleavage of carbon-carbon bond in 1,2 glycols or their amino alkyl amino derivatives to form dialdehydes. These dialdehydes forms complex with pararosaniline dye to produce magenta colored chromogen indicating the presence of glycosylation.

Reagent

Preparation of Schiff Reagent

1.0 g of basic fuschin powder was dissolved in 200 ml of boiled water (temperature maintained at 50°C) and stirred well until the dye dissolves completely. Then 20 ml of 1N HCl was added and kept for maturation for 24 hrs in dark. After this, 2 g of activated charcoal powder was added and then filtered and sorted in brown colored bottle.

Procedure

25 µg of purified 88 kDa protein was resolved in 12.0 % SDS PAGE and PAS staining was done to identify whether the protein was glycosylated? In brief, the gel was rinsed with 7.5 % acetic acid for 10 min and then incubated in 1.0 % periodic acid for 15 min at 4°C. Then the gels were rinsed with distilled water briefly followed by incubation with Schiff reagent (Sigma Chemical Company, MO, U.S.A) for 15 min at 4°C. After this step the gels were rinsed in 0.5 % sodium metabisulfate for 20 min. The reaction was stopped

by addition of 2.5 % acetic acid and the gels were then stored under water. Albumin was used as positive control.

Amino Terminal Acid sequencing of 88 kDa protein (Matsudaria, 1987)

The purified protein was subjected to 12 % SDS PAGE electrophoresis and electrotransfered to polyvinylidine difluoride membrane (PVDF, Millipore Co., Bedford, MA, U.S.A) using Bio Rad Trans blot equipment. The electrotransfer was performed in buffer containing (3.3 g Tris base, 14.4 g glycine, 1.0 g SDS, 800 ml dH_2O and 200 ml CH_3OH) for 1hr at 100 V/400mA. The transferred protein was visualized by staining the membrane with 0.1 % ponceau S (dissolved in 1.0 % acetic acid in water). The protein band was then carefully excised, de-stained with several changes of milli Q water. Then the PVDF membrane containing the protein was directly subjected to N terminal sequencing by using automated Edman degradation chemistry (Matsudaria, 1987) in Applied Biosystems 470A gas phase amino acid sequencer (Foster City, CA, USA). The derived sequence was analyzed by protein and DNA data base analysis using SWISS Prot, and NCBI programs.

Determination internal peptide sequence

250 µg of purified protein was precipitated with 300 µl ice cold 100 % TCA and left in ice for 45 min. Then centrifuged at 4000 rpm at 4°C for 15 min. The supernatant was removed carefully and discarded. The precipitate was washed twice with ice cold acetone (200 µl) by centrifuging as described above. Finally the precipitate was dissolved in 300 µl of 8 M urea solution. The solubilized protein was reduced with 10 µl of 45 mM dithiothreitol (DTT). This procedure was done in sealed ampoules containing sample under nitrogen gas at 52°C for 30

min. After this step, the 10 µl of 25 mM iodoacetamide was added, after cooling the tubes to room temperature. The reaction was allowed to take place at room temperature for 15 min. Then to this 150 µl of milli Q water was added. The protein was then digested with trypsin at the concentration of 0.2 mg / ml in 20mM ammonium carbonate buffer pH 8.0 for 12 – 16 hrs at 37°C. The contents were freezed rapidly in liquid nitrogen to stop the reaction and the contents were reduced to near dryness in rotary speed vacumn concentrator (Savant Instruments, U.S.A). The residue was finally dissolved in 100 µl of milli Q water and injected in to C_8 column (Phenomax, U.S.A) and the peptides were separated suing RP HPLC (Shimadzu –SLC6A, Japan). The solvents used were as follows **A**: 0.1 % TFA in water. **B**: 80 % CH_3CN in 0.75 % TFA in water. The gradient program was: 0-5 min 0 % B, 5 – 65 min 100 % B, 65-70 min 100 % B, 80 min 0 % B. Elution was monitored at 214 nm. The major peaks were collected and used for further processing. Data collection and processing was done using C-R7A Plus software (Shimadzu Corp. Japan). The volume of the major fractions was reduced to 50 µl and then rechromatography was performed to obtain pure peptides. The chromatographic conditions were similar as described above. Then the fractions containing pure peptides were reduced in volume and then loaded on to automated amino acid sequencer (PSQ –1, Shimadzu, Japan) to obtain the internal peptide sequence. The derived sequence was analyzed by protein and DNA data base analysis using SWISS Prot, and NCBI programs.

Biochemical characterization studies

88 kDa protein was associated with Eales' disease where ocular inflammation exists. 88kDa protein was presumed to be a stress/acute phase protein and thus tested for any functional property for transferrin, ceruloplasmin and haptoglobin. Transferrin activity in the purified

protein was done by measuring iron (Carter, 1971) and iron binding capacity (ICSH, 1978). The results were expressed as µg of iron dL^{-1} serum. Ceruloplasmin activity was examined by its oxidation of p-phenylendiamine (Ravin, 1961). Haptoglobin activity was assessed by its agglutination property on RBCs (Herbert, 1978).

While performing the titration of H_2O_2 with $KMnO_4$, addition of 88kDa protein reduced the level of H_2O_2, hence glutathione peroxidase activity was suspected and it was tested by the method of Rotruck et al. (1973). As 88kDa protein has anti TBARS activity, it was thought to have antioxidant property. The protein was thus tested for superoxide dismutase (SOD) activity by following the method of Mishra and Fridovich (1972).

Ceruloplasmin activity for 88 kDa protein

Principle

Ceruloplasmin (copper oxidase) catalyses the oxidation of some polyamines and its action on p-phenylenediamine was used as a measure of the amount present in the sample.

Reagents

1. Acetate buffer: 0.1M, pH 6.0. 10.0 ml of 0.1 M acetic acid (0.57 ml glacial acetic acid diluted with water to 100 ml) was added to 200 ml of sodium acetate. The pH was adjusted with 0.1 N acetic acid.
2. Sodium azide : 0.1 % in 0.1 M acetate buffer.
3. P- phenylenediamine dihydrochloride : 0.25 % in 0.1 M acetate buffer.

Procedure

2.0 ml of buffer was added to 1 ml of p-phenylenediamine dihydrochloride reagent for the test and 1.0 ml of buffer and 1 ml p-phenylenediamine dihydrochloride reagent along with 1.0 ml of sodium azide was added for blank. The tubes were kept at 37°C for 10 min and then 10 µg of purified 88 kDa protein was added to each tube and mixed well. The optical density of the test and blank tubes were read at 530 nm exactly 10 min after addition of the sample. Ceruloplasmin content was expressed as mg/dl by using a conversion factor 0.05.

Determination of transferrin activity of 88 kDa protein

Principle

By decreasing the pH of the serum, Iron was released from transferrin. Fe (III) is reduced to Fe (II) then Fe (II) complexes with bathophenanthroline which absorbs at 534 nm.

Reagents

1. Protein precipitant: 10 g TCA , 3 ml of thioglycolic acid and 0.2 ml of concentrated HCl was added and made up to 100 ml with dH_2O.
2. 2 M sodium acetate.
3. Chromogen solution: 20 mg bathophenanthroline sulfonate in 100 ml of 2M sodium acetate solution.
4. 2 N H_2SO_4 : 7.2 ml con H_2SO_4 made up to 100 ml with dH_2O.

Procedure

2.0 ml of buffer was added to 1 ml of p-phenylenediamine dihydrochloride reagent for the test and 1.0 ml of buffer and 1 ml p-phenylenediamine dihydrochloride reagent along with 1.0 ml of sodium azide was added for blank. The tubes were kept at 37°C for 10 min and then 10 µg of purified 88 kDa protein was added to each tube and mixed well. The optical density of the test and blank tubes were read at 530 nm exactly 10 min after addition of the sample. Ceruloplasmin content was expressed as mg/dl by using a conversion factor 0.05.

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2. 2 M sodium acetate.
3. Chromogen solution: 20 mg bathophenanthroline sulfonate in 100 ml of 2M sodium acetate solution.
4. 2 N H_2SO_4 : 7.2 ml con H_2SO_4 made up to 100 ml with dH_2O.

5. Iron standard (stock solution): 7.02 mg ferrous ammonium sulfate was dissolved in water containing 0.2 ml of 2N H_2SO_4 . The solution was made up to 100 ml with dH_2O.
6. Working standard : 40 ml stock diluted to 100 ml with dH_2O (concentration of iron 400 µg / 100 ml)

Procedure

To 1ml serum, either 1 µg or 2 µg of purified 88 kDa protein was added and incubated for 15 min at 37°C. Then 1.0 ml water and 2.0 ml protein precipitant are added. Mixed thoroughly and allowed to stand for 5 minutes and centrifuged at 3000 rpm for 10 – 15 min. To 2.0 ml supernatant, 2.0 ml chromogen solution was added. Mixed well and allowed to stand for 5 minutes. The absorbance was measured at 534 nm. A standard curve was constructed using, 0.4 µg – 8.0 µg iron from working standard and treating them similar to test sample. Amount of iron was expressed as µg % / dL of serum.

Iron binding capacity

Reagents

Stock standard $FeCl_3$: 145 mg of $Fecl_3$ in 100ml of 0.5N HCl.

Working standard $FeCl_3$: 1.0 ml of stock solution was diluted to 100 ml with dH_2O.

Procedure

To 1.0 ml of serum either 1 or 2 µg of purified 88 kDa protein was added and incubated at 37°C for 10 min. Then 4.0 ml of working ferric chloride and 1ml of water was added

mixed well. Following this step 400 mg of solid magnesium carbonate was added and vigorously cyclomixed for 30 to 60 minutes. Then centrifuged for 30 min at 3000 rpm to get a clear supernatant.. 2.0 ml of the supernatant was taken and to this added 2 ml of protein precipitant and allowed to stand at RT for 10 minutes. Then centrifuged again for 10 minutes. 2.0 ml of supernatant was taken and added 2.0 ml of chromogen reagent was added and allowed to stand at RT for 5 minutes. The absorbance was measured at 535 nm, against blank containing 2.0 ml distil water and 2ml of chromogen reagent. The iron binding capacity was expressed as µg % /dL of serum.

Determining glutathione peroxidase activity for 88 kDa protein

Reagents

1. Sodium phosphate buffer 0.32 M pH 7.0.
2. EDTA solution (0.8 mM) in water
3. Sodium azide solution (10 mM)
4. GSH (20 mg %)in water.
5. H_2O_2 (2.5 mM) in water.
6. 10 % TCA in water
7. Disodium hydrogen phosphate solution (0.3M)
8. DTNB solution: 20 mg DTNB was prepared in 1.0 % sodium citrate solution.

Procedure

0.2 ml of EDTA, sodium azide, glutathione and hydrogen peroxide together with 0.5 ml buffer and 0.7ml water were mixed together in a test tube and e pre-incubated at 37°C for 10 minutes for temperature equilibration. 10 µg of purified 88 kDa protein was added

and incubated again at RT for 15 min. Similarly control tube consisted all the reagents with 88 kDa protein but 1.0 ml of 10 % TCA was added to arrest the reaction. After incubation, 1.0 ml of 10 % TCA was added to the test and centrifuged at 3000 rpm for 15 – 20 min. Then to the supernatant, 8.0 ml of phosphate solution and 1.0 ml of DTNB reagent was added and the absorbance was read immediately at 412 nm against water blank. GPx activity was expressed as μM GSH utilized / min / mg protein.

Determination of SOD activity for 88 kDa protein

The procedure followed is similar as described for RBCs, except the omission of extraction step. 25 μg of purified 88 kDa protein was used for the assay.

Determination of haptoglobin activity of 88 kDa protein

Procedure

Human 'O' positive blood was obtained from healthy volunteer. The blood was collected in EDTA anticoagulant tube. 1.0 ml of whole blood was washed with 0.9 % NaCl by centrifuging at 2000 rpm for 10-15 min. Then the packed cells free of plasma components were used for the assay. In brief, 100 μl of packed cells were diluted in 1.0 ml of 0.9 % NaCl. 100 μl of diluted packed cells were incubated with 10 – 50 μg of purified 88 kDa protein in final volume of 150 μl in microtitre plates and incubated at RT for 2 hrs. Then the agglutination activity was observed using hand held magnifier lens.

Production and purification of polyclonal antibody against 88 kDa protein

1.5 mg of purified 88kDa protein was used for raising polyclonal antibody in goat as described earlier (Chase,1967). The antibody was custom produced by Chemicon Inc.,

(Temecula, CA, USA). Purification of IgG from polyclonal antiserum was performed by adopting the method described by (Talwar, 1967). 50.0 ml of antiserum was diluted with equal volume of 0.2M Tris-HCl buffer pH 8.0. Then this was salt fractionated with 18 % sodium sulfate. This step was followed by centrifugation at 7000 rpm for 15 at 4°C. Supernatant was discarded and the precipitate was again dissolved in 20 ml of 0.1M Tris-HCl buffer pH 8.0 and this was followed by 15 % sodium sulfate fractionation. After centrifugation, the precipitate was again dissolved in 0.1M Tris-HCl buffer pH 8.0. The salt was removed by dialysis against Milli Q water overnight in cold room.

This fraction was taken and used for further purification process. Separation of immunoglobulins was done by ion exchange chromatography, as it exploits the differences in net charge between various classes of immunoglobulins. In this study diethylaminoethyl (DEAE) cellulose was used. At pH 8.0, most proteins would have net negative charge, while the resin would have positive charge. Amongst immunoglobulins, at pH 8.0, IgG is weekly charged followed by IgA and IgM in the order of increasing net negatively charges. Hence the resin was equilibrated with 0.01M PBS pH 8.0 and then after application of the sample, the elution was done using aforementioned buffer, which permits a condition, where IgG gets eluted as unbound fractions. The fractions were dialyzed against Milli Q water and concentrated using sucrose. Sucrose was removed using molecular weight cut-off filter (Centricon, 10 kDa, Millipore, U.S.A). Then the partially purified goat anti human 88kDa IgG was stabilized in 10 mM PBS pH 7.4 with 0.01 % sodium azide as preservative and then stored in frozen aliquots.

Immunoelectrophoresis

Immunoelectrophoresis was performed using the method described by Culliford (1964) and Lowrell (1972). Briefly, 1 % agarose was dissolved in 0.04 M Veronal buffer pH 8.6. The same buffer was used as running buffer. Electrophoresis was carried out for 2 hrs with 10 mA/slide. Following electrophoresis, another horizontal trough was made in between the two antigen wells and filled with 0.1ml of partially purified anti 88 kDa antibody (1:500). The slides were then kept in a humid chamber at room temperature for 24 to 36 hrs to allow the passive diffusion of antigen and antibody. Precipitin arcs were viewed directly under dark back ground, the slides were then dried completely (60°C for 2 hrs) and subsequently stained with coomassie brilliant blue (R250) for permanent documentation.

Western blot analysis

The purified 88 kDa protein (20 μg) from serum and vitreous was subjected to 12 % SDS PAGE. Then the proteins were electrotransfered to nitrocellulose membrane (Millipore Co., Bedford, MA, U.S.A) as described earlier. The membrane was blocked with 5 % skimmed milk in PBS pH 7.4 for 1hr at RT in a rocking platform. The membrane then probed with anti 88kDa antibody (goat polyclonal 1:500dilution) for 2-3 hrs at RT. Following this step, mouse anti goat IgG antibody (Sigma, St Louis, CA, USA. 1:1000 dilution) was added and the membranes were incubated for 2 hrs at RT. The protein was visualized using nitrotetrazoliumbluechloride (NBT) / 5-bromo 4- chloro 3-indoyl phosphate (BCIP) substrate solution (Bangalore Geni Pvt Ltd., India). The protein bands appeared as deep purple colour.

Immunolocalization of 88 kDa protein in ERM of patients with ED

Four surgically excised ERM from six patients with ED were used for immunohistochemical analysis to find out the precise cellular location of 88 kDa protein. The tissues were processed and embedded in paraffin wax blocks. 5 µm sections were cut using microtome (Leica, JUNG RM 2045, Germany) and the paraffin sections were transferred on to poly-L-Lysine coated glass slides. After deparaffinization, the sections were incubated with 3 % H_2O_2 for 10 min to quench the endogenous peroxidase activity. The sections were washed in PBS pH 7.4 and exposed to 2.0 % normal goat serum solution (Dako, Copenhagen, Denmark) for 30 min at 4°C. Sections were then washed in PBS and then probed with primary antibody, anti 88 kDa (goat polyclonal, 1: 200) for 2 hrs at room temperature in humid chamber. After this step, the sections were washed in PBS and then incubated with biotinylated rabbit anti goat IgG antibody (Dako) for 45 min, followed by incubation with avidin-biotin complex (Dako) for 15 min at room temperature.

Final colour was developed with 3,3' diaminobenzidine (DAB) substrate kit (Dako). For histologic correlation, the sections were counter stained in haematoxylin for 45 seconds, then slides were coverslipped and mounted (Entellan, Merk, Darmstadt, Germany). Photographic documentation (ASA 100 film, Eastman Kodak, Rochester, NY, U.S.A) was performed with an optical microscope (Nikon, Japan) at X 200 and X 400 magnifications respectively.

Screening of serum samples for the presence of 88 kDa protein in ocular and systemic inflammatory diseases which mimic ED

25 µg serum protein from was loaded on to 7.5 % NPAGE. The gels were stained with 0.025 % coomassie brilliant blue R250 and destained with 40 % methanol and 7 % acetic acid. The protein profiles in the gels were then documented using a gel documentation system (Image Master, VDS, Amersham Biosciences, UK).

Western blot analysis to investigate the immunological identity of 88 kDa present in ED and other diseases

25 µg of protein was subjected to 7.5 % native NPAGE. Resolved proteins on the gels were then electrotransfered to PVDF membrane (Pharmacia Biosciences, UK). The membrane was blocked with 5 % skimmed milk in phosphate buffered saline (PBS) pH 7.4 for 1 hr at room temperature. Subsequently the membranes were probed with anti 88 kDa antibody (goat polyclonal, affinity purified, 1: 500 dilution) and incubated at room temperature for two hours. After this step, the membranes were again washed in PBS pH 7.4 and then incubated with anti goat IgG antibody conjugated with alkaline phosphatase was used as secondary antibody (Santa Cruz Biotechnology, CA, U.S.A, 1:1000) for an hour and the protein was visualized using NBT/BCIP substrate solution (Banglore Geni Pvt Ltd. India).

Immunolocalization of 88 kDa in tissue sections of skin lesions obtained from patients with leprosy and tuberculosis.

Four tissues from patients with tuberculosis and leprosy were processed and embedded in paraffin wax blocks. 5 µm sections were cut using microtome (Leica, JUNG RM 2045, Germany) and the paraffin sections were transferred on to poly-L-Lysine coated glass slides. After deparafinization, the sections were incubated with 3.0 % H_2O_2 for 10 min to

quench the endogenous peroxidase activity. The sections were washed in PBS pH 7.4 and exposed to 2 % normal goat serum solution (Dako, Copenhagen, Denmark) for 30 min at 4°C. Sections were then washed in PBS and then probed with primary antibody, anti 88 kDa (goat polyclonal, 1: 200) for 2 hrs at room temperature in humid chamber. After this step, the sections were washed in PBS and then incubated with biotinylated rabbit anti goat IgG antibody (Dako) for 45 min, followed by incubation avidin-biotin complex (Dako) for 15 min at room temperature. Final colour was developed with 3,3' diaminobenzidine (DAB) substrate kit (Dako). For histologic correlation, the sections were counter stained in haematoxylin for 45 seconds, then and slides were mounted (Entellan, Merk, Darmstadt, Germany) and coverslipped. Photographic documentation (ASA 100 film, Eastman Kodak, Rochester, NY, U.S.A) was performed with an optical microscope (Nikon, Japan).

Statistical analysis

All values were expressed as mean ± standard deviation. Wherever appropriate, Student's t test, one-way analysis of variance (ANOVA) and Pearson's correlation test were employed to assess the statistical significance of the data. $P < 0.05$ was considered statistically significant.

CHAPTER 1

CHAPTER 1

Role of Hydroxyl Radical in promoting Retinal Vasculitis and Neovascularization in Eales' Disease

Introduction

Inflammation is the main clinical feature in ED (Spitzans et al., 1975). Earlier, histopathological studies on epiretinal membranes (ERM) obtained from ED revealed the infiltration of chronic inflammatory cells around the newly formed blood vessels (Elliot., 1954; Kono et al., 1985; Badrinath et al., 1992; Biswas and Rao, 1990). Intact internal limiting membrane (blood retinal barrier) serves as a barrier to the migration of inflammatory cells from circulation into the retina. However, during inflammatory conditions, the blood retinal barrier breaks leading to increased vascular permeability associated with migration and invasion of circulating inflammatory cells into the retina (Lightman and Greenwood, 1992). Immunophenotyping of inflammatory cellular infiltrates in ERM in ED were found to contain mononuclear macrophages (Badrinath et al., 1992; Biswas and Rao, 1990).

$OH^{\bullet}$ can be produced by two mechanisms: they can be either produced through constitute expression of ROS metabolizing enzymes (for e.g. xanthine oxidase (XOD), phagocyte oxidase (Phox), Cycloxyginase (COX) by the endothelium or by induced expression of iNOS in phagocytes recruited to the inflammed site. Phagocytes play an important role during inflammatory conditions; they protect the host from invading pathogens by killing them with toxic oxygen metabolites. However the same metabolites might be detrimental

to the host tissue, if there is a defect in the antioxidant capacity (Babior, 2000). Owing to high reactivity $OH^{\bullet}$ readily attacks lipids, proteins and nucleic acids leading to the loss of their structure and functions (Moskovitz et al., 2002). Present study describes the role of $OH^{\bullet}$ generation in MC and its possible involvement with the development of RV in patients with ED.

$OH^{\bullet}$ has short half-life of 10^{-9} Sec.; therefore its measurement becomes extremely difficult (Cheeseman and Slater, 1993). In order to circumvent this problem, Electron Spin Resonance Spectrometric (ESR) technique has been employed in this study to identify and quantitate $OH^{\bullet}$ in MC of patients with ED (Yaamazake and Piette, 1990). MC was used as the clinical material because, the cluster differentiating antigen present on these cells (CD11b) and the mononuclear macrophage infiltrates seen in ERM were found to be same (Badrinath et al., 1992; Biswas and Rao, 1990). Moreover, leukocytes play an important role in the tissue remodeling during inflammatory conditions by its ability to produce and modulate the ROS activities (Fantone and Ward, 1982).

Brief introduction to ESR

ESR or electron paramagnetic resonance spectroscopy (EPR) is a technique used extensively to detect and identify many short - lived free radical species. ESR spectroscopy depends upon the interaction of an external homogenous magnetic field with the magnetic moment of an unpaired electron within a free radical molecule. A single unpaired or free electron, which has an spin quantum number (M_s) of $\pm$ ½ , assumes two orientations in the magnetic field. The two orientations of the free electron

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at different energy levels, where the difference in energy is referred to as the Zeeman splitting. As the magnetic field (H) increases the magnitude of the Zeeman splitting also increases. The difference between the two energy levels is expressed in the following equation: $\mathbf{h\nu = g\beta HM_s}$, where H is the external magnetic field (approx. 3500 Gauss), h is Planck's constant, β is the Bohr magneton or the magnetic moment of the electron, g is the g-value characteristic for a given unpaired electron system (approx. 2), M_s is the spin quantum number ($\pm$½) and ν is the frequency of the electromagnetic radiation, i.e. the microwave region of $10^9 - 10^{11}$ Hz (approx. 9.5GHz for X- band spectroscopy).

The unpaired electron can also interact with a neighboring nucleus, further modifying the energy level by a given amount, defined as the hyperfine splitting constant (a), depending on the spin quantum number (I). The hyperfine splitting constant is characteristic of the interaction between the unpaired electron and the nucleus of a given molecule. The separation of lines, known as hyperfine splitting, is the most useful characteristic analytical feature of an ESR spectrum, which can be used for structural determination of free radicals. The number of resonant lines observed in ESR spectrum can be predicted by the following relationship : $\mathbf{2nI + 1}$, where I is the spin of the nucleus and n is the number of magnetically equivalent nuclei with spin I. The sensitivity or detection limit of ESR spectrometers is on the order of ~10^{-8}M. Direct detection of free radicals is possible only when the free radicals are present or formed in high concentrations. However, very few radicals, especially those involved in biological systems, have sufficiently long enough life-times to maintain a steady state concentration of radicals above the threshold of ~ 10^{-8} M.

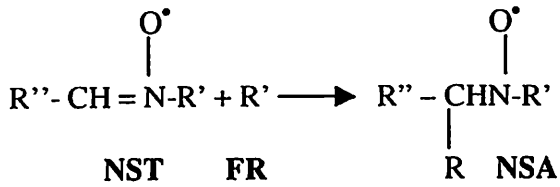
Spin Trapping

The spin traping method was developed to extend the limits of ESR spectroscopy so that lower concentrations of free radicals could be detected indirectly. This method involves the trapping of reactive short-lived free radicals by a diamagnetic spin trap compound via an addition reaction to produce a more stable free radical or spin adducts. The spin aduct that is formed is paramagnetic and has an ESR spectrum with a hyperfine splitting constant and g-value characteristic of the type of free radical trapped. Thus, the structure of the radical trapped can usually be deduced, although the most difficult aspect of the spin trapping technique is the correct assignment of the nitroxide spectrum to the original radical species.

The most commonly used spin trapping agents in biological systems are nitrone derivatives. Examples of commonly used nitrones are α-phenyl-N-tert butyl nitrone (PBN); 5,5-dimethyl-1-pyrroline-N-oxide (DMPO); and α - (4-pyridyl-1-oxide)-N-tert-butyl nitrone (POBN). The nitrone spin trap react with the free radical species via a carbon located in a beta-position relative to the nitrogen (see figure below). The hydrogen (I = ½) that is tow bonds away from the nitroxyl radical functional group is referred to as the beta-hydrogen. The appearance of the pattern in ESR is dependent on the magnitudes and the degree of overlap between, the nitroxyl nitrogen and the beta-hydrogen hyperfine splitting constants.

The magnitude of the beta-hydrogen splitting (aH) in the nitroxide spin adduct is influenced by the bulk of the attached free radical. In general, small R groups have a large

aH value. Solvents can have major effect on the hyperfine splitting observed for a spin adducts. In general, increases in solvent polarity produce an increase in the nitrogen – hyperfine splitting as the spin density of the nitrogen increases.



NST- Nitrone spin trap; FR – free radical; NSA- Nitroxide spin adduct

DMPO is the most widely used spin trap for the study of oxygen-centered free radicals, due to its solubility in water, its rapid diffusion in biological systems, and the formation of stable adducts. The more lipophilic character of PBN limits its solubility in aqueous environment, but allows it to readily cross membranes and diffuse into cells. As a result of its lipophilicity, PBN has been extensively sued to trap lipid -derived radicals. The more water-soluble PBN analogue, POBN due to its elevated hydrophilicity and its ability to permeate cell membranes it can also be used as an alternative to DMPO to trap and identity the oxygen – centered radicals. In this study POBN has been used to detect the $OH^{\bullet}$ in monocytes of patients with ED.

Results

Hydroxyl radical generation by MC in vitro

Addition of PMA (100 ng / ml) to MC in the presence of 4- POBN and ethanol in chelex treated HBSS, pH 7.4 resulted in the detection of $OH^{\bullet}$ with hyperfine constants (A_N = 15. 75 G, A_H = 2.45 G). Results showed enhanced production of $OH^{\bullet}$ (two fold) in MC of patients with ED, when compared with healthy control subjects. The results are shown in (Table 1, Fig.1).

Effect of iron chelators on hydroxyl radical generation and TBARS formation

Since iron plays an important role in generation of $OH^{\bullet}$ by Fenton reaction, the possible effect of iron chelators on the $OH^{\bullet}$ generation and TBARS accumulation in MC of patients with ED were investigated. To test this hypothesis, MC (pooled) from ED were incubated with or without desferrioxamine and DTPA along with PMA. Results revealed that when MC was co-incubated with PMA (100 ng) and 25 μM DTPA, there was 76 % inhibition in $OH^{\bullet}$ generation (Table 2, Fig. 2B). When the concentration of DTPA was increased to 50 μM, there was 96 % inhibition in $OH^{\bullet}$ generation (Table 2, Fig. 2C).

On the other hand, when MC was co- treated with PMA (100 ng/ml) and desferrioxamine 25 μM there was 73 % inhibition in $OH^{\bullet}$ generation (Table 2, Fig. 3B), 91 % inhibition in the presence of 50 μM desferrioxamine (Table 2, Fig. 3C). TBARS levels were significantly reduced (six fold) when MC of patients with ED were co-

Table 1: Levels of oxidants and antioxidant parameters in MC of patients with Eales' disease and healthy control subjects

Sl. No	Variables	Healthy control (n=12)	Eales' disease (n=12)	*P* value
1.	$OH^{\bullet\bullet}$	1.95 ± 0.81	5.44 ± 0.81	< 0.001
2.	SOD[¶]	7.54 ± 1.29	3.18 ± 0.51	< 0.001
3.	Iron[§]	0.52 ± 0.11	2.76 ± 1.11	< 0.001
4.	Zinc[§]	0.62 ± 0.15	0.307 ± 0.12	< 0.05
5.	TBARS[‡]	6.58 ± 2.89	18.59 ± 7.0	< 0.001

All values are mean ± SD

Age/sex: Eales' disease – 34 ± 7 years, all Males
Healthy Control Subjects – 31 ± 8 years, all Males

• - Peak intensity (cm)
¶ - Units/mg protein
§ - µg/mg protein
‡ - nmole MDA/mg protein

Table 2: Effect of DTPA and desferoxammine on $OH^{\bullet}$ production in PBMM from patients with Eales' Disease.

SL. No.	Treatment	$OH^{\bullet}$ peak intensity (cm)
1.	None	6.2 ±0.52
2.	DTPA (25 µM)	1.42 ± 0.17^{a}
3.	DTPA (50 µM)	0.25 ± 0.09^{b}
4.	Desferoxammine (25 µM)	1.9 ± 0.05^{c}
5.	Desferoxammine (50 µM)	0.11 ± 0.08^{d}

Values are mean ± SD of 3 independent experiments.
a,b,c,d ; $P < 0.001$ with respect to none.

Table 3: Effect of iron chelators in lipid peroxide index in PBMM from patients with ED.

Sl. No.	Treatment	TBARS (nmole MDA/mg protein)
1.	None	23 ± 4
2.	50 µm DTPA	1.42 ± 1.2^{a}
3.	50 µm Desferoxamine	2.7 ± 1.8^{b}

Values are mean ± SD of 3 independent experiments.
a,b; $P < 0.001$ with respect to none.

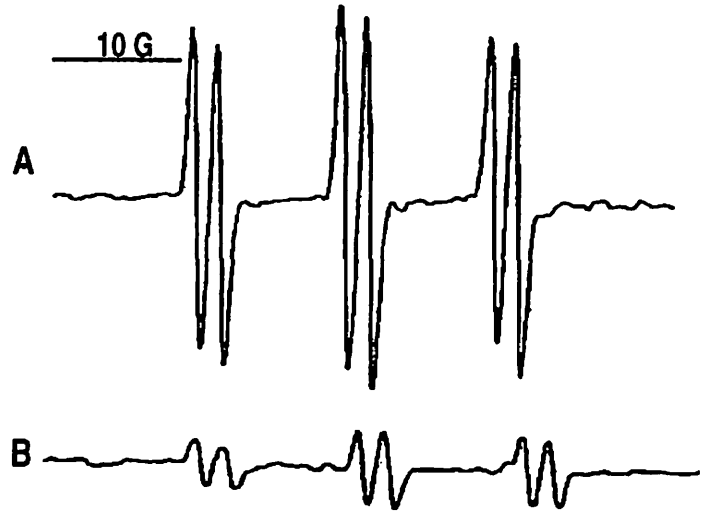


Figure 1. $OH^{\bullet}$ generation in MC *in vitro*. ESR spectrum 15 min after addition of PMA (100 ng / ml) to MC from ED. (B). ESR spectrum 15 min after addition of PMA (100 ng / ml) to MC from healthy control subjects.

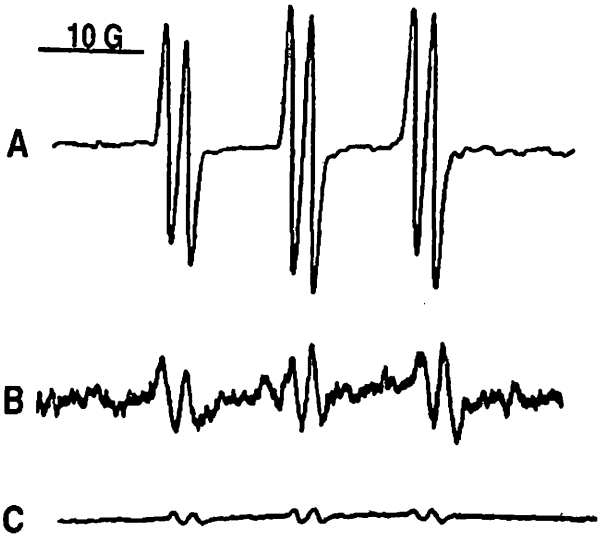


Figure 2. Effect of DTPA on $OH^{\bullet}$ generation in MC from ED. (A) ESR spectrum 15 min after addition of PMA (100 ng / ml) without DTPA. (B) same as A with addition of 25 μM DTPA. (C) same as B but with 50 μM DTPA

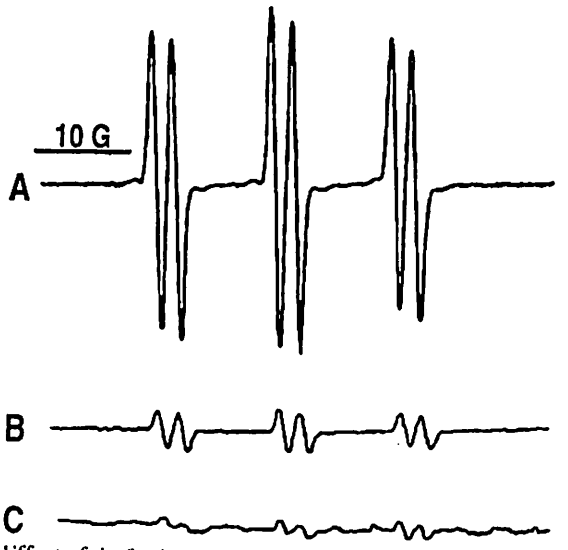


Figure . 3 Effect of desferrioxammine on OH• generation in MC from ED. (A) ESR spectrum 15 min after addition of PMA (100 ng/ml) without desferrioxammine. (B). Same as A with 25 μM desferroxamine. (C) same as B with 50 μM desferrioxammine

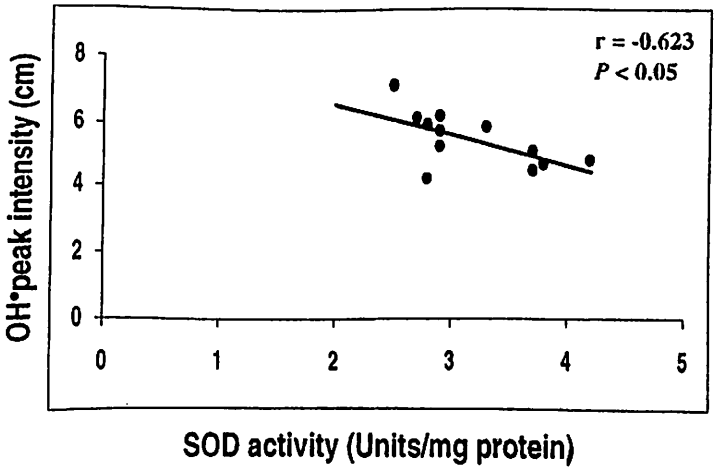


Figure 4A. Correlation between hydroxyl radical production and SOD activity in MC of patients with ED.

incubated with PMA (100 ng / ml) and 50 μM DTPA (Table 3). Similarly TBARS formation was inhibited by 8.5 fold when the cells were treated with 50 μM desferrioxamine, when compared with cells not treated with any of the above iron chelators (Table 3).

Correlation between hydroxyl radical generation with SOD activity, TBARS accumulation and iron content in MC of patients with ED

Increase in $OH^{\bullet}$ generation and diminished SOD activities in MC obtained from patients with ED were mutually correlated (Fig. 4A). Similarly, positive correlation was observed with increased $OH^{\bullet}$ production and iron levels in MC of patients with ED (Fig. 4B). In addition to the above findings, enhanced $OH^{\bullet}$ production correlated with TBARS accumulation in MC from patients with ED (Fig. 4C). There was no significant correlation observed between the variables in MC obtained from healthy control subjects.

Diminished SOD activity and increased lipid peroxidation in ED

SOD activity was diminished by two fold and TBARS accumulation was three folds higher in MC of patients with ED, when compared with healthy control subjects. Iron level was elevated by five- fold and zinc levels was decreased by two fold in MC with ED when compared with control subjects. The results are shown in (Table 1).

Plasma GSH levels

The plasma levels of GSH were significantly low 1.5 ± 0.25 in patients with ED than in healthy control subjects 2.9 ± 0.7 ($P < 0.001$)

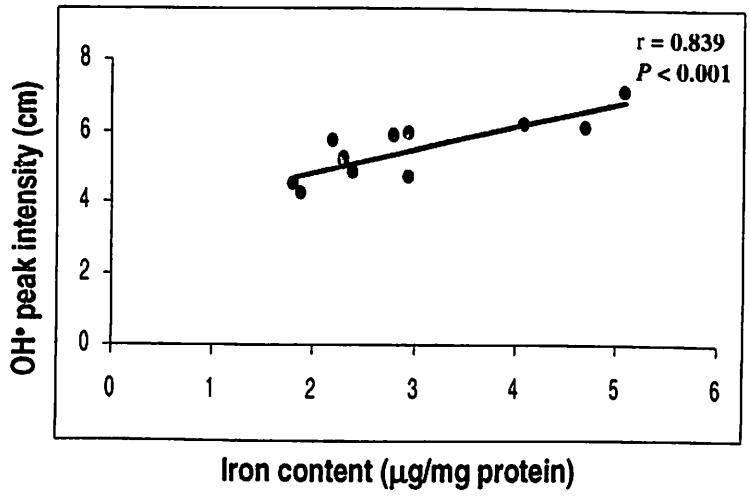


Figure 4B. Correlation between hydroxyl radical production and iron content in MC of patients with ED.

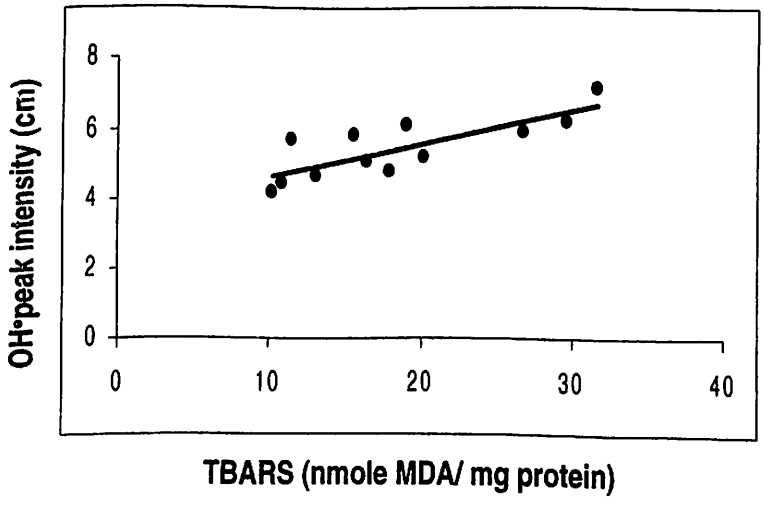


Figure 4C. Correlation between hydroxyl radical production and iron content in MC of patients with ED.

Indirect measurement of inflammation

Patients with ED has significantly higher leukocyte counts (10200 ± 400 cells/mm^3) than normal healthy control subjects (6800 ± 1200 cells/mm^3 , $P < 0.001$), however this were within the upper quartile of the accepted normal range.

In vitro Viability of MC

A significant ($P < 0.001$) reduction in ED, MC viability after an incubation of 1 hr in ED serum was evident when compared with 1 hr incubation of normal control MC in normal control sera. In fact, normal control sera promoted the survival of ED MC ($P < 0.001$), whereas ED sera significantly killed normal control MC ($P < 0.001$) (Fig. 5).

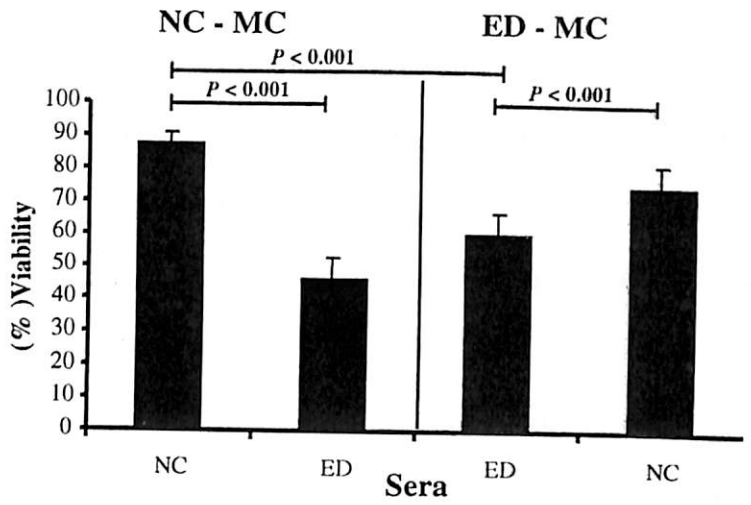


Figure. 5 In vitro viability of normal control and ED MC incubated for an hour in autologous and heterologous ED and control sera. Viability is expressed as the ratio of cell counts before and after incubation with sera as (%). Data are mean ± SD, n = 12

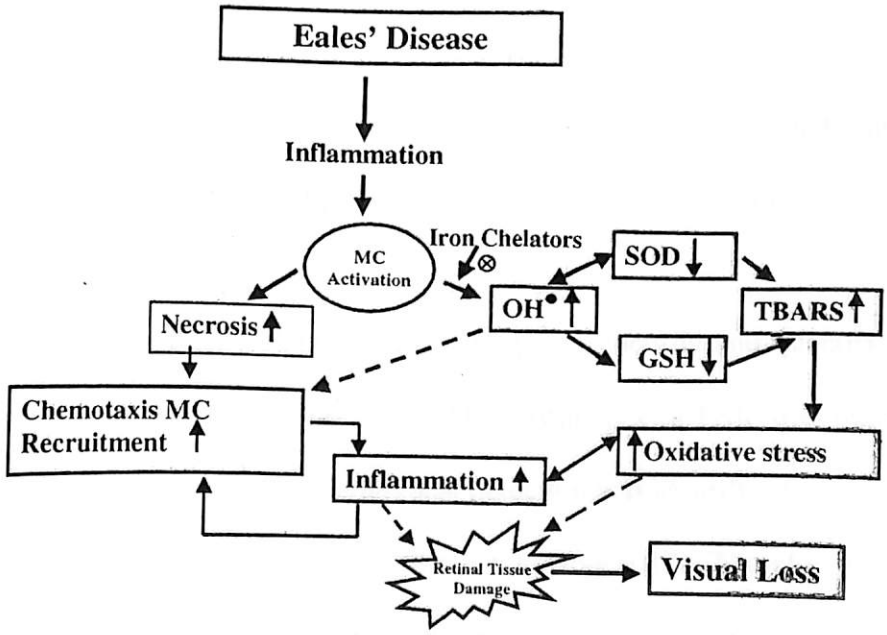


Figure 6. Schematic representation for the role of hydroxyl radical in promoting retinal vasculitis and neovascularization in Eales' disease

Discussion

Oxidative stress has been shown to be involved in the pathophysiology of intra ocular inflammatory diseases (Rose et al., 1998). Chronic intraocular inflammation is a major cause of blindness (Rao, 1990 & 2000). The loss of vision is the result of damage inflicted by the inflammatory cell infiltration in to the retina (Rao, 1990 & 2000).

The hydroxyl radical is highly reactive and it reacts with variety of macromolecules such as, lipids, proteins and DNA leading to lipid peroxidation, protein aggregation and DNA hydroxylation. All these events result in the loss of structure and function of the above bio-molecules (Beckman et al., 1990). $OH^{\bullet}$ reacts with biological molecules in diffusion - controlled fashion. Since their half- life in cells has been estimated to be 10^{-9} sec, their measurement becomes very difficult (Beckman and Ames 1998). Two trapping methods, which were developed with great potential for specifically identifying $OH^{\bullet}$ are (i) ESR trapping (Rosen and Klebanoff, 1979) and (ii) aromatic hydroxylation (Kaur and Halliwell, 1994). Also ESR has been used to demonstrate and establish the oxidative stress in-patients with type 2 diabetes mellitus (Beauvieux et al., 1998).

In the present study ESR technique was employed to identify and quantify $OH^{\bullet}$ upon stimulation of MC with mitogenic agent. The advantages of ESR spin trapping in the analysis of $HO^{\cdot}$ are (i) provides unequivocal detection and identification (ii) the assay is very sensitive fast and the (iii) trapped spin is relatively stable. Therefore the analysis of radical becomes simple, because the propagation of free radical chain reaction are eliminated, when the MC were stimulated with PMA and 4- POBN. We detected POBN

nitroxide adduct with hyperfine splitting constants (A_H = 15.75 G, A_N = 2.45 G) consistent with those reported previously for the α hydroxylation adducts of 4 – POBN (Kennedy and Mason, 1990; Yamazaki and Piette, 1990).

Reactive oxygen species (ROS) has been shown to enhance the migration of monocytes across the blood – retinal barrier (BRB) in uveitis and optic neuritis (Rao et al., 1994; Guy et al., 1989,1993). In addition to the above, ROS has been shown to be involved in monocyte chemotaxis across blood brain barrier and implicated in the pathogenesis of neuro degenerative diseases (Goes et al., 2001; Glabinsky et al., 1993).

As mentioned earlier, in ED also there is disruption of BRB and infiltration of circulating phagocytes in to the retina. Hence in this study, the properties and activities of MC and their possible contributions to oxidative stress and inflammation in patients with ED was studied. When separated, PMA - stimulated ED MC produced significantly higher amounts of $OH^{\bullet}$, when compared with normal control MC (Table 1). The increase in $OH^{\bullet}$ generation in ED MC, paralleled with diminished SOD activity (Fig. 4A), increased iron content (Fig. 4B).

The major detriment for the cellular toxicity of $O_2^{\cdot-}$ and H_2O_2 is the availability of iron to catalyze $OH^{\bullet}$ production through Fenton reaction (Ramos et al., 1992). The production of free radicals is essential to the host to combat the invading pathogens. However, this can be deleterious if their toxicity is not controlled by intra / extracellular defense mechanisms (Halliwell and Gutridge, 1985).

A range of intracellular antioxidant defense systems limits the toxicity ROS. SOD is of particular interest, because this enzyme is involved in detoxification of superoxide anion produced by xanthine oxidase and phagocyte oxidase (Halliwell and Gutridge 1985). SOD has been shown to protect iron-sulfur clusters in the active sites of enzymes involved electron transport chain (Srinivasan et al., 2000). Under oxidative stress conditions it has been shown that ROS inactivates SOD by oxidizing some of the crucial residues involved in the catalytic reaction, which leads results in loss of its activity (Keyer and Imlay, 1996). As a result of this, iron is released from iron-sulfur complexes. This gives autonomous power for iron to participate in Fenton reaction to produce $OH^{\bullet}$, which does the damaging role during oxidative stress conditions (Nunoshina et al., 1999 ; Morris et al., 1995).

Zinc is an integral part of SOD, which is needed to maintain its structural and functional integrity (Firdovich, 1995). Zinc depletion can also result in diminished activity of SOD (Firdovich, 1995) Several studies are available for the beneficial role of zinc as antioxidant and ameliorating oxidative stress in various animal models and human clinical trials reviewed in detail by (Powell, 2000). In the present study we have found positive correlation with zinc deficiency and diminished SOD activity in MC of patients with ED (data not shown). Therefore, this could be another reason for finding diminished SOD activity in patients with ED.

Iron chelators have been shown to ameliorate oxidative stress in animal models of systemic and ocular inflammations by inhibiting $OH^{\bullet}$ production (Blake et al., 1983 ;

Guttridge et al., 1979; Rao et al., 1986). In the present study treatment of iron chelators inhibited the formation of $OH^{\bullet}$ and thus preventing the formation of TBARS in ED, which were consistent with earlier findings of (Blake et al., 1983; Guttridge et al., 1979; Rao et al., 1986). Recently Zhang and Frei (2003) has demonstrated that chelation of intracellular iron inhibits the expression of TNF-α and adhesion molecules in cultured human arotic endothelial cells. All these reports suggest the significance of iron chelation in controlling oxidative stress induced tissue damage.

Plasma GSH levels were low in ED (Table I). Negative linear correlation was also observed with enhanced $OH^{\bullet}$ in MC versus diminished plasma GSH levels in patients with ED ($r = -0.571$; $P = 0.04$; $n = 12$). The decrease in plasma GSH in ED, may be an outcome of greater GSH consumption by ROS generated by phagocytes during respiratory burst induced by inflammatory conditions (Thomas et al., 1988 ; Seres et al 2000). The important outcome of this study is demonstrating the involvement of MC in oxidative stress and inflammation in ED. The leukostatic effect of the ED sera suggests that extracellular factors were involved in the cellular priming of ED - MC. Primed MC die by self- necrosis and at the same time, they actively recruit more MC into circulation. Reports are available, pertaining to the determination and use of antioxidant and oxidant parameters MC as predictive and prognostic marker in systemic inflammatory conditions such as myocardial infraction and atherosclerosis (Swirski et al., 2001; Phillips et al., 1992). There is concomitant increase in MC recruitment and impairment in the antioxidant defense system. This results in the enhanced free radical generation in MC, which amplify the inflammatory cascade leading to the retinal tissue damage in patients

with ED. The interpretations of the results from this study are schematically represented in (Fig. 6).

To conclude, by using EPR it was possible to demonstrate the pivotal role of $OH^{\bullet}$ generation in MC and its possible contribution in the development of RV in ED. Hence clinical trial is recommended to test the efficacy of vitamin E, C and desferrioxamine in controlling RV and NV in patients with ED.

CHAPTER 2

CHAPTER 2

Role of Reactive Nitrogen Species in promoting Retinal Vasculitis and Neovascularization in Eales' Disease.

Introduction

Phagocyte generated ROS and RNS have been implicated in the tissue damage during various systemic diseases (Patel et al., 1999). There is considerable evidence that reactive oxygen species (ROS) and RNS, particularly peroxynitrite ($ONOO^-$) can act as an important mediator of inflammation, shock and ischemia/reperfusion injury (Hanafy et al., 2001). In diseases where role of ROS/RNS has been associated its pathogenesis, selective inhibition of iNOS activity or supplementation with antioxidants which quench these radicals, have been shown to ameliorate the inflammation induced tissue injury (Hobbs et al., 1999). Till date there are no reports in literature about human clinical research done on the role of ROS/RNS in development of RV in ED to date.

Hence, the aim of the present study was to investigate whether accumulation RNS is involved in the development of retinal vasculitis (RV) damage in ED. The rational of using MC was briefed in the previous chapter. In addition to the above MC harbors higher amounts of iNOS and they have been shown to play a major role in tissue remodeling during inflammation by its ability to produce and modulate the ROS/RNS activities (Gugon et al., 1998).

Results

Increased iNOS expression in MC of ED

Western blot analysis of iNOS in MC indicated that the expression of iNOS was increased by (~ 5-fold, $P < 0.001$, Fig. 1A) in patients with ED when compared with control subjects (Fig. 1 B).

NTYR accumulation

Standardization of 3NYTR determination by RP-HPLC – UV detection

Fig. 2A shows the amino acid profile in the cell lysate. Fig 2B depicts typical chromatogram for authentic standard of 3NTYR. Fig. 2C depicts 3NTYR chromatogram in healthy control subjects while Fig. 2D represents the 3NTYR chromatogram in MC of patients with ED.

3NTYR in ED

Table 2 describes 3NTYR levels in MC of patients with ED and control subjects. Accumulation of 3 NTYR in patients with ED were 2.5 fold higher when compared with control subjects ($P < 0.001$).

SOD activity

The levels of antioxidants and oxidants parameters studied in MC of patients with ED and control subjects are given in (Table 1). MC - SOD levels were decreased by 2- fold ($P < 0.001$) in patients with ED when compared with control subjects (Table 1).

Table 1: Levels of antioxidant and oxidant parameters determined in MC of patients with Eales' Disease and control subjects.

Parameters	Healthy control subjects (n = 20)	Eales' Disease (n = 35)	P value
SOD (Units/mg protein)	7.28 ± 0.31	3.75 ± 0.58	<0.001
TBARS (nmole MDA/mg protein)	11.15 ± 1.67	24.76 ± 2.18	<0.001
Iron*	0.44 ± 0.11	0.72 ± 0.18	<0.001
Copper*	0.38 ± 0.12	0.78 ± 0.21	<0.05
Zinc *	0.62 ± 0.15	0.41 ± 0.09	<0.05

All values are mean ± S.D.

Age/sex: Eales' disease – 31 ± 11 years, all Males.

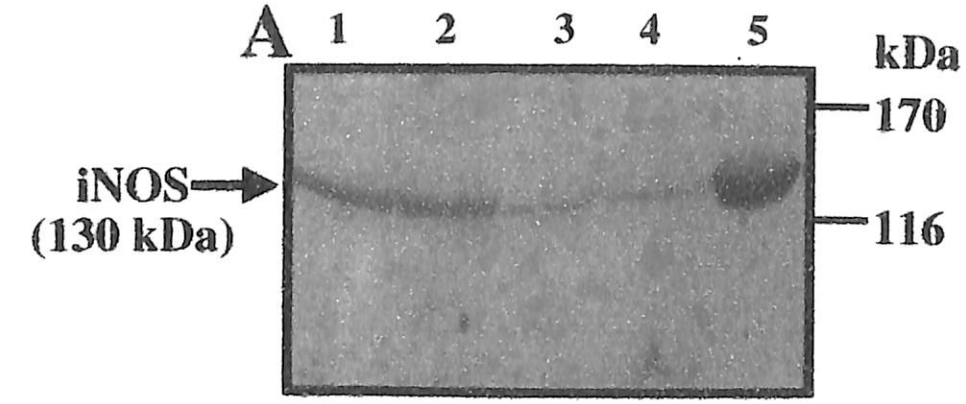
Healthy Control subjects – 29 ± 11 years, all Males

* Levels of these trace elements were expressed as µg /mg Protein.

Table 2: Levels of Tyr and 3 NTYR in MC lysates of patients with Eales' disease and control subjects.

Parameters	Control Subjects (n = 20)	Eales' Disease (n = 35)	P value
Tyr (µg/ml)	16.2 ± 6.6	18.9 ± 6.2	NS
3 NTYR (µg /ml)	0.38 ± 0.14	1.08 ± 0.39	< 0.001
Tyr (µg / mg protein)	4.68 ± 2.3 4.	43 ± 1.5	NS
3 NTYR (µg /mg protein)	0.10 ± 0.04	0.24 ± 0.10	< 0.001
3 NTYR /Tyr	0.021 ± 0.014	0.054 ± 0.03	< 0.001

All values are mean ± SD. NS - Not significant.



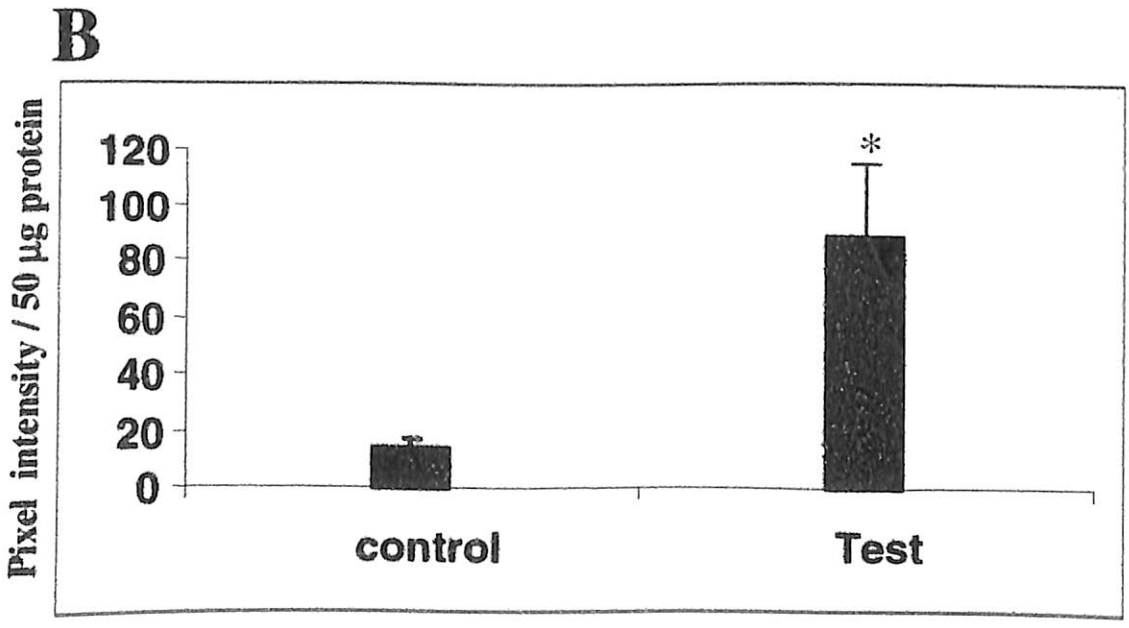


Figure 1. Western blot analysis of iNOS protein in MC. (A). Immunoblot shows the expression of iNOS protein. Lane1 a and 2: cell lysates from patients with Eales' disease. Lane 3 and 4: cell lysates from healthy control subjects. Lane 5: RAW 264.7 cell line stimulated with lipopolysaccharide was used as positive control. (B) Quantification of iNOS protein expression in MC of control subjects and patients with Eales' disease. Values are expressed as mean ± SD, * $P < 0.001$

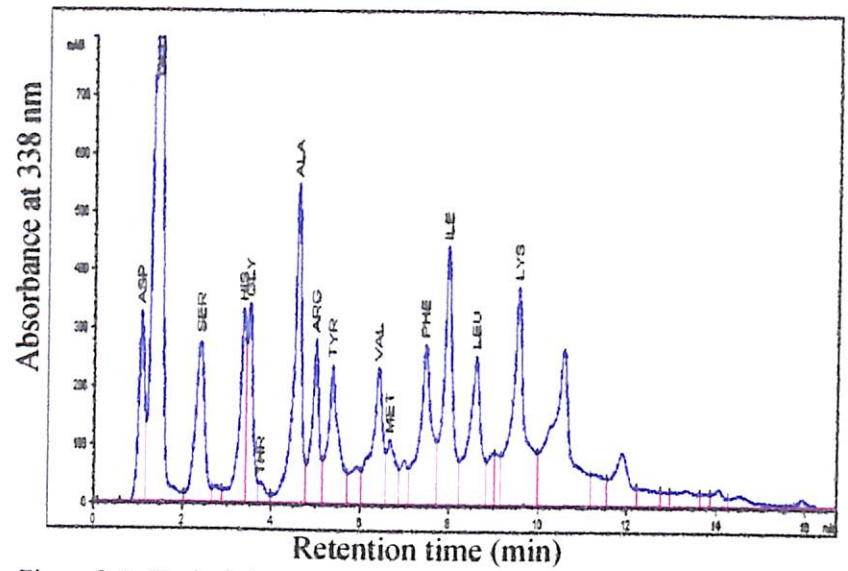


Figure 2 A. Typical chromatogram of MC cell protein hydrolysate. The separation of amino acids are depicted.

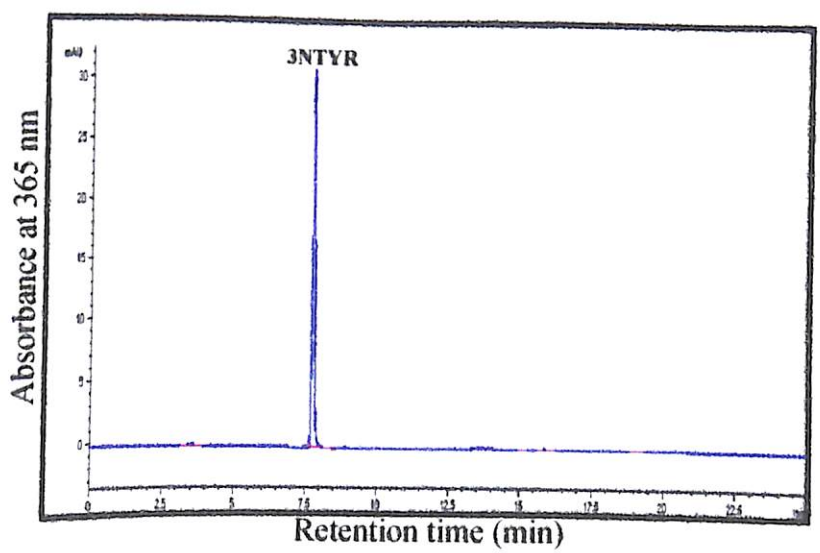


Figure 2B. Chromatogram representing the authentic 3NTYR standard (10 pmole/L). 3 NTYR had retention time of 7.6 min.

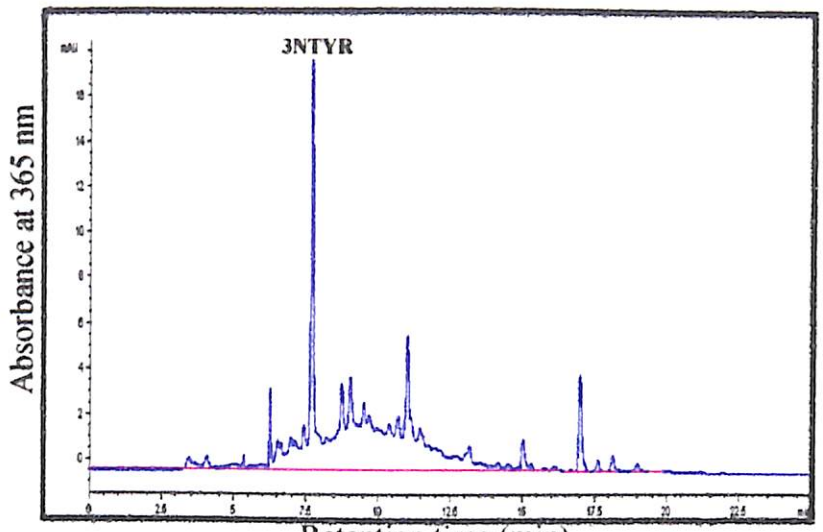


Figure 2C. Chromatogram showing the sample spiked with 5 pmole/L 3NTYR to identify its RT in clinical sample.

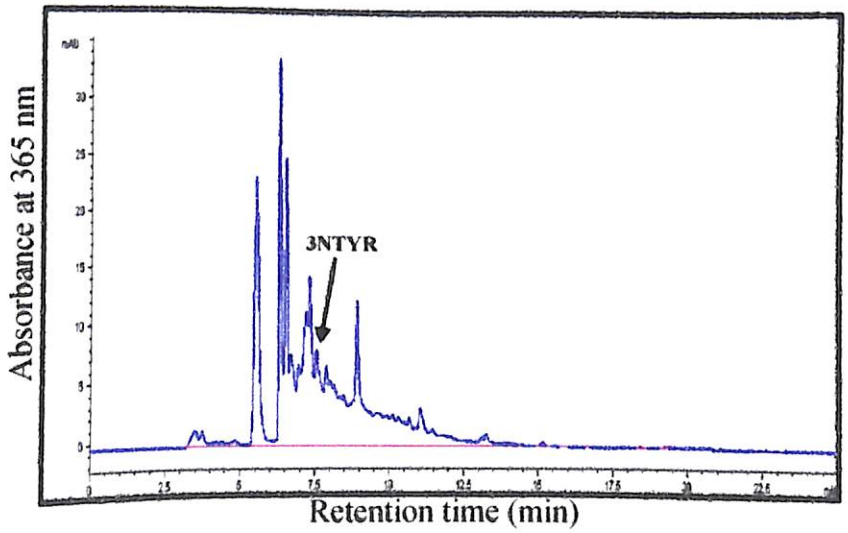


Figure 2D. Chromatogram representative of 3NTYR in control sample

TBARS accumulation

Results indicated that the patients with ED have 2-fold ($P < 0.001$) increase in the accumulation of TBARS in their MC when compared with healthy controls (Table 1).

Trace elements

We observed 1.7, 2.0 and fold increase ($P < 0.05$) in level of iron and copper in MC of patients with ED (Table 1). Zinc levels were diminished by 1.5 fold ($P < 0.001$) in MC of patients with ED, when compared with healthy volunteers.

Immunolocalization of iNOS and NTYR

In order to correlate the changes that was observed in MC of patients with ED, whether it was really consistent with the disease process? immunolocalization experiments were performed in the ERM, from the patients with ED. Strong immunoreactivity for iNOS (Fig. 3 B) and NTYR (Fig. 3 C) were observed in the MC, lymphocytes and endothelium, in all the eight ERM studied.

Correlation analysis for the variables

Correlation analysis of the variables studied in MC of patients with ED are presented in (Table 3). Increased iNOS expression and 3NTYR accumulation were positively correlated. Increased iNOS expression correlated with diminished SOD activity and increased lipid peroxidation in MC of patients with ED. Elevated levels of iron and copper, positively correlated with accumulation of lipid peroxides in MC of patients with ED. Similarly decreased zinc content in MC correlated with diminished SOD activity in patients with ED.

Table 3: Correlation between diminished antioxidant levels and accumulation of ROS and RNS in MC of patients with Eales' disease.

Variables	r	*P*
iNOS Vs 3 NTYR	0.42	< 0.05
3 NTYR Vs SOD	- 0.39	< 0.05
iNOS Vs SOD	- 0.31	< 0.05
iNOS Vs TBARS	0.38	< 0.05
SOD Vs TBARS	0.39	< 0.05
Fe Vs TBARS	0.31	< 0.05
Cu Vs TBARS	0.38	< 0.05
Zn Vs SOD	0.63	< 0.001

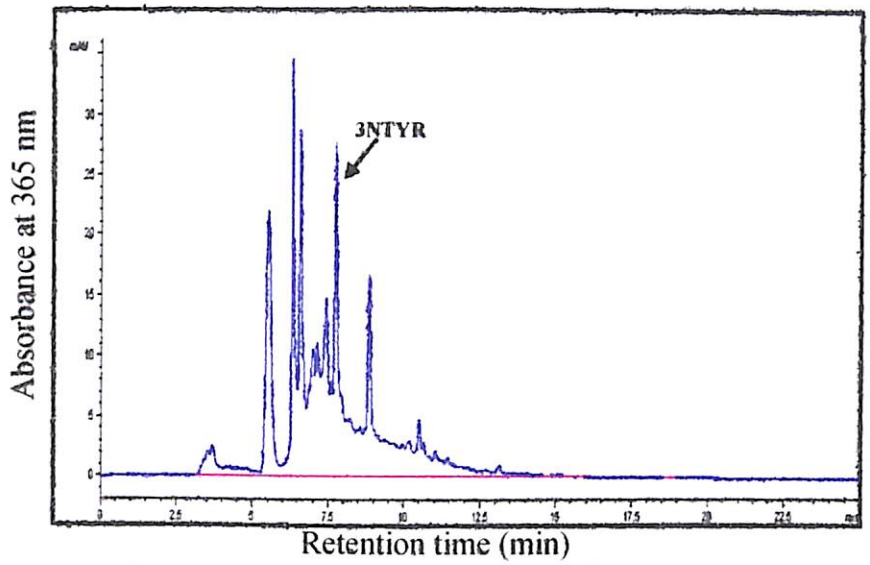


Figure 2E. Chromatogram representative of 3NTYR in Eales' disease sample

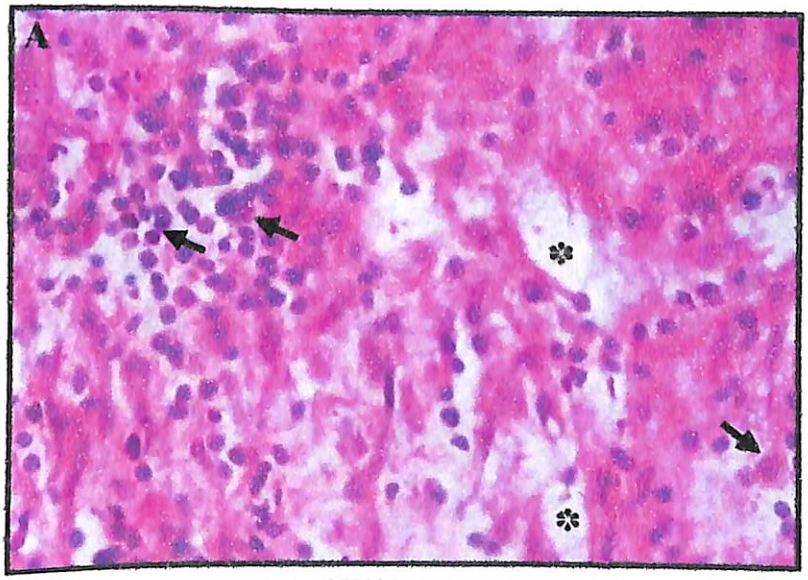


Figure 3A. Haematoxylin Eosin staining of ERM from patients with ED. The neovascular channels are indicated by bold asterisk. Leukocyte infiltration in indicated by arrow (X 200)

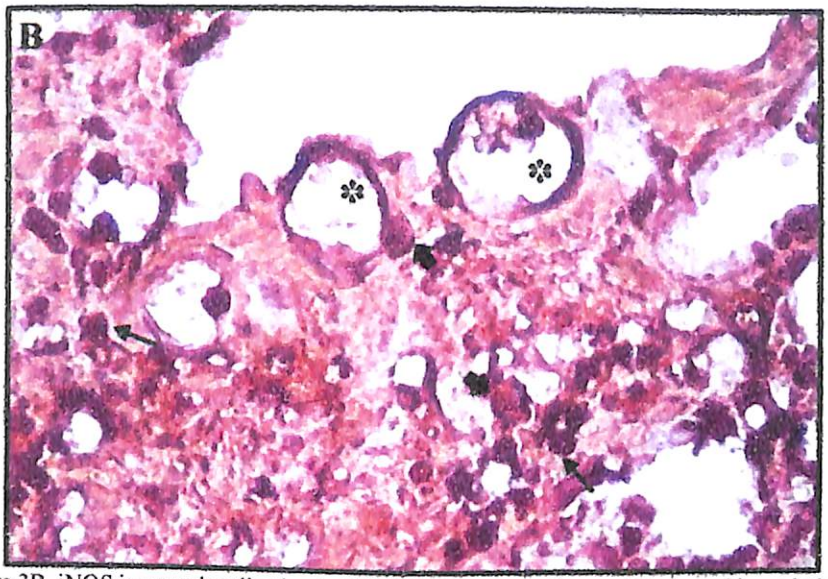


Figure 3B. iNOS immunolocalization in ERM. Strong expression could be noted in endothelium (asterisk) and in the lymphocytes (long arrow) and monocytes by bold arrow (X 400)

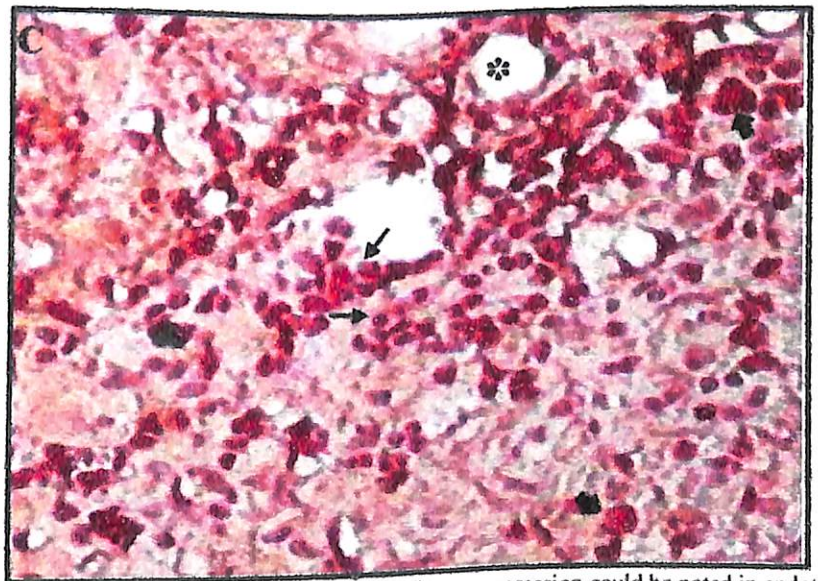


Figure 3C. 3NTYR immunolocalization in ERM. Strong expression could be noted in endothelium (asterisk) and in the lymphocytes (long arrow) and monocytes by bold arrow (X 200)

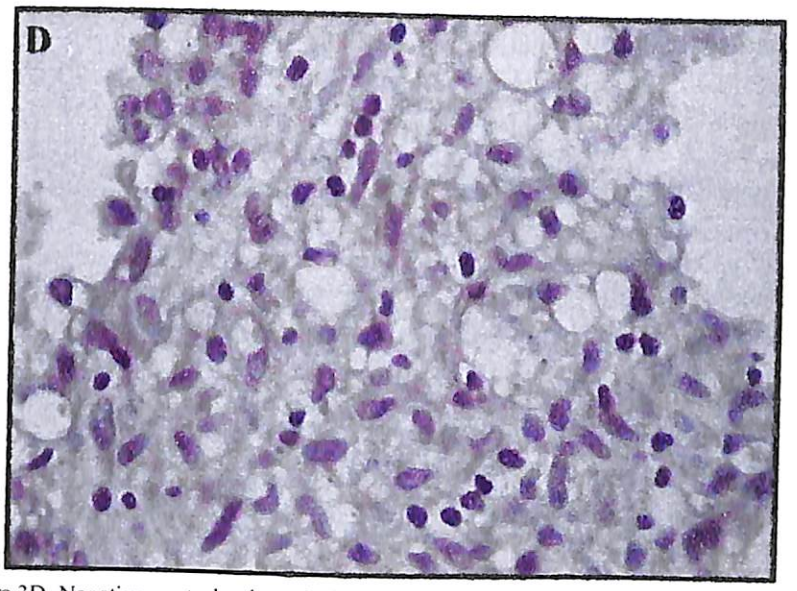


Figure 3D. Negative control, where the immunoreactivly for iNOS and 3NTYR in ERM of patients with ED, was abolished by omission of primary antibody

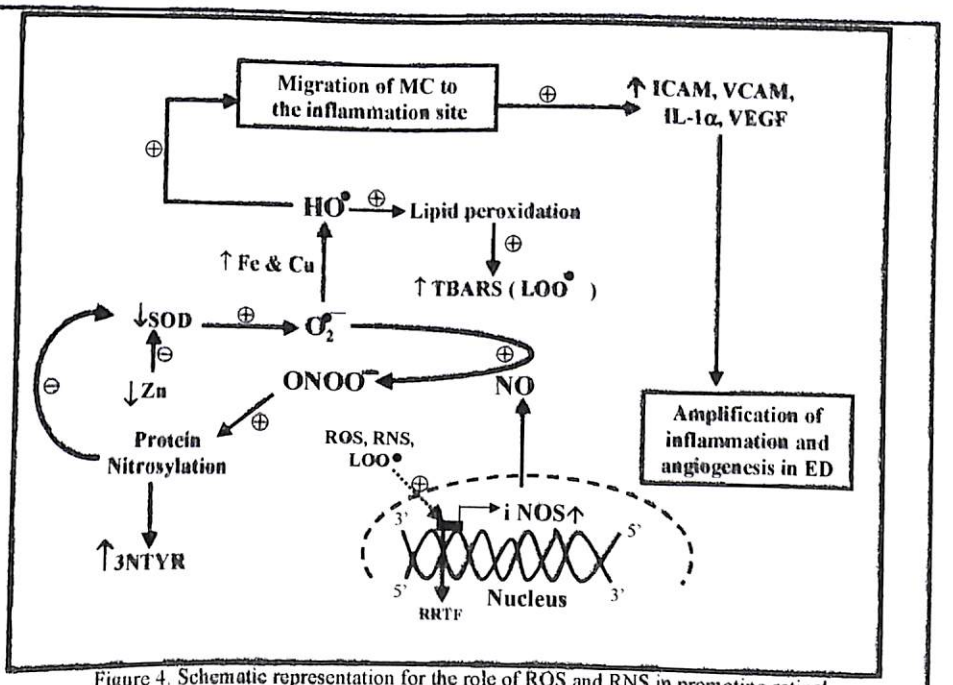


Figure 4. Schematic representation for the role of ROS and RNS in promoting retinal vasculitis and neovascularization in Eales' disease

Discussion

iNOS expression can be triggered by variety of inflammatory mediators, ROS and RNS (Kröncke et al., 1995). The salient aspect of iNOS is that once activated, it remains active for days and continues to produce NO (Mair et al., 1994). iNOS has also got the ability to modulate the acute and chronic inflammatory conditions (Laroux et al., 2000). This is supported by the findings that NO accumulation is found to be higher in the inflammed tissue (Ischiropoulos, 1998). In the present study strong immunoreactivity was observed for iNOS in leukocytes and in the endothelial cells in ERM obtained from patients with ED (Figure 2B).

These were consistent with the reports published by other investigators in various inflammatory conditions (Ischiropoulos, 1998). Recently Yilmaz et al., (2002) have reported elevated levels of NO in aqueous humor obtained from patients with Behçet disease (triad of dermatitis, genital ulceration and intraocular inflammation). Increased iNOS expression, diminished SOD activity and NTYR accumulation have been reported in retinal tissues obtained from animal model of uveitis (Wu et al. 1997). In addition to the above, increased lipid peroxide and NO levels were associated with the pathogenesis of age related macular degeneration (ARMD) (Toten et al., 2001). In the present study, strong relationship has been observed between increased iNOS expression and lipid peroxidation in the MC of patients with ED (Table 5).

Superoxide anion ($O_2^{\bullet-}$) reacts with NO to produce peroxynitrite ($ONOO^-$) (Huie and Padmaja, 1993). The reaction of NO with $O_2^{\bullet-}$ is facilitated during inflammatory conditions, since phagocytes have been shown to simultaneously generate NO and $O_2^{\bullet-}$ at

a similar rate (Rodenas et al., 1995). $ONOO^-$ is considered powerful oxidant than $O_2^{\bullet-}$, because the former has the higher diffusion coefficient and half-life than the latter (Huie and Padmaja, 1993). $ONOO^-$ on entering the cell rapidly nitrates variety of macromolecules chiefly at the aromatic ring (Beckman et al., 1996). RNS and ROS has been shown to trigger the expression of proinflammatory cytokines such as interleukin 1 (IL - 1β) tumor necrosis factor (TNF - α) and adhesion molecules such as intracellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM), through redox regulated transcription factors (RRTF) such as NF-κB , AP-1 and SP-1 (Ou et al., 1997; Zhang and Frei, 2003). These events result in the adhesion of leukocytes to the endothelium, which amplifies the inflammatory cascade and promotes tissue damage. However, one needs to determine the levels of ICAM and VCAM in vitreous of patients with ED.

Nitration has been shown to affect the structure and function of various macromolecules, which includes SOD (Yamakura et al., 1998). This may also be the other reason for diminished SOD activity observed in MC of patients with ED. The diminished SOD activity correlated with increased iNOS expression and NTYR accumulation (Table 5). Zn is considered to be vital component of SOD, which maintains its structural and functional integrity. Zn deficient rats have been shown to diminish SOD activity and longevity (Powell, 2000). These effects were reverted, when the animals were supplemented with diet rich in Zn. Taking these observations into account, similar reason postulated for observing diminished SOD activity in patients with ED.

Recently (Savvides et al., 2002) has demonstrated the inactivation of antioxidant enzyme glutathione reductase by $ONOO^-$. The accumulation of 3NTYR in protein is considered to be the stable " foot print" of RNS stress (Radi et al., 2001). Increased accumulation of 3NTYR has been reported in various pathological conditions (Radi et al., 2001). Immunolocalization studies for NTYR in ERM of ED, revealed strong immunoreactivity in infiltrating leukocytes and in the endothelial cells, suggesting that our findings were consistent with the disease process. Moreover, positive correlation was observed between iNOS expression and NTYR accumulation in MC with that of ERM obtained from ED which showed immunoreactivity for iNOS and 3NTYR ($r = 0.914$; $P < 0.001$).

Diminished SOD activity results in the accumulation of $O_2^{\bullet-}$. $O_2^{\bullet-}$ have been shown to release iron and copper from active sites of enzymes involved in respiratory chain (Flint et al., 1993). Increased level of Fe and Cu catalyzes the production of hydroxyl radical ($OH^{\bullet}$) via Haber-Weiss Fenton reaction (Harris et al., 1995). $OH^{\bullet}$ induces lipid, protein and DNA oxidation. These oxidation products have been shown to be elevated in various inflammatory diseases, aging, neurodegenerative diseases, diabetes and other diseases (Harris et al., 1995). In this study, we have observed increased iron and copper levels in MC of ED correlating with elevated lipid peroxides in patients with ED.

Both $O_2^{\bullet-}$ and $ONOO^-$ have been shown to be strong chemoattractant for phagocytes (Goes et al., 2001). These reactive species have been shown to involved in initiating, propagating and amplifying tissue damage during inflammatory conditions by their ability to modulate the expression of inflammatory cytokines in the phagocytes (Goes et

al., 2001). In Behçet's disease, where intraocular inflammation is one of the clinical feature, free radical mediated tissue damage has been proposed by many investigators and these reports clearly revealed the role of free radicals in the disease process (Niwa et al., 1982 ; Dogan et al., 1994; Köse et al., 1995 & 2001). Antioxidant supplementation has been shown to be beneficial delaying clinical symptoms, in diseases were the role of ROS/RNS are associated with the disease process (McCall and Feri, 1999). Recently Köçkam and Naziroğlu (2002) have reported the beneficial effect of vitamin E supplementation in delaying the clinical symptoms in patients with Behçet's disease. Like wise oral supplementation of vitamin C and E has been shown to improve the visual acuity in patients with uveitis (Rooji et al., 1999). In addition to the above, selective inhibition of iNOS expression or supplementation of antioxidant vitamins, has been shown to ameliorate inflammatory tissue damage in uveitis (Morell et al., 2002). The possible role of RNS in promoting RV in ED is outlined in Fig.4.

To summarize, results from this study revealed potential involvement of RNS and ROS in the development of RV in patients with ED. Considering the potential involvement of free radicals in the disease process, it is predicted that selective inhibition of iNOS and or supplementation of vitamin E or C might be beneficial to patients with ED in controlling retinal tissue damage.

CHAPTER 3

CHAPTER 3

Carbonyl Content as Biomarker for Oxidative Protein damage in Eales' Disease

Introduction

Free radicals produced during oxidative stress can damage the peptide backbone, resulting in the generation of protein carbonyls. The process is initiated by hydrogen abstraction from α-carbon in a peptide chain. If two protein radicals are in close proximity, they may cross-link with one another by radical coupling (Berlett and Stadtman, 1997). Alternatively, oxygen can attack the α-carbon centered to form peroxide intermediates, leading to rearrangements and subsequent cleavage of the peptide bond to form carbonyl-containing peptides (Stadtman, 1995; Gebicki et al., 1993). Protein carbonyls also may be generated by the oxidation of several amino acid side chains (e.g., Lys, Arg, Pro) and by the formation of Michael adducts between nucleophilic residues and α,β -unsaturated aldehydes (Fu et al., 1998).

The pathway for the formation of carbonyl groups is schematically represented in Fig.1 Carbonyl contents are analyzed by the reaction of 2,4-dinitrophenylhydrazine with proteins to form the corresponding hydrazone (Levine et al., 1990). Protein peroxidation in contrast to lipid peroxidation, does not have the features of chain reaction. The oxidized proteins are selectively removed by proteinases and detoxified. However most often they escape from degradation and they accumulate in extracellular fluids and intracellularly, where they module the gene expression of molecules involved in inflammatory reaction whereby exerting proinflammatory stress. Such modifications have

been shown to affect the structure and function of several structural and non-structural proteins (Stadtman and Berlett, 1998). The plasma proteins damaged by free radicals have long half-life. Therefore, evaluation of carbonyl content is considered to be potential biomarker to assess the redox status in physiological and pathological conditions. Increased accumulation of carbonyl groups has been reported in several human diseases (Devein et al., 2000). However there are no reports available in ED, hence in the present study, carbonyl content was determined to assess the free radical mediated protein damage and its possible association with the disease process.

Results

Table 1 describes the levels of protein carbonyl groups content along with other antioxidant/oxidant parameters studied in patients with ED and control subjects. Protein carbonyl contents were 3.4 and 1.8 fold, higher in AV stage and HV stage in patients with ED, when compared with healthy control subjects (Tables. 1 & 2). Increase of carbonyl content in AV and HV stages of ED correlated with diminished SOD activity and plasma GSH content (Tables. 3 & 4).

Plasma GSH content was decreased by 2.8 and 2 fold in AV and HV stages of ED, when compared with healthy control subjects (Tables. 1 & 2). SOD activity were diminished by 1.8 and 1.2 fold in AV and HV stages of ED, when compared with control subjects (Tables. 1 & 2). TBARS accumulation were 1.8 and 1.4 fold higher in AV and HV stages of ED when compared with healthy control subjects (Tables. 1 & 2). Decreased GSH levels correlated with increased carbonyl content in ED (Tables 3 & 4).Similarly,

Table 1: Levels of protein carbonyl groups content in plasma and antioxidant status in patients with Eales' disease and healthy control subjects

Group	Plasma Carbonyl content (nmole/mg protein)	Plasma GSH (μM)	SOD (Units /g Hb)	TBARS (nmole MDA/g Hb)
Active perivasculities (n=20)	2.6 ± 0.61	1.4 ± 0.54	2388 ± 651	45.71 ± 6.7
Healed perivasculities (n=15)	1.4 ± 0.11	1.9 ± 0.41	3543 ± 142	37.26 ± 4.13
Healthy Control (n=20)	0.76 ± 0.21	3.9 ± 0.11	4293 ± 245	25.51 ± 2.71

All values are Mean ± S.D

Age / sex

Active Vasculitis: 26 ± 4.8 years, all Males.

Healed Vasculitis : 27 ± 5.5 years , all Males.

Healthy Control subjects : 24.4 ± 5.23 years, all Males.

Table 2: Schefee Post Hoc test for evaluating the statistical significance for the variables studied between groups.

Comparison	Carbonyl content (nmole / mg protein)	Plasma GSH (μM)	SOD (Units /g Hb)	TBARS (nmole MDA / g Hb)
Active perivasculities*	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$
Healed perivasculities*	$P < 0.001$	$P < 0.001$	$P < 0.05$	$P < 0.001$
Active perivasculities Vs Healed perivasculities	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

* Comparison with respect to healthy controls subjects.

Table 3: Correlation between accumulation of carbonyl groups and antioxidant parameters in AV stage in patients with Eales' disease.

Variables (n=20)	r	P
Carbonyl Content Vs Plasma GSH	- 0.6031	< 0.05
Carbonyl Content Vs SOD activity	- 0.8305	< 0.001
SOD activity VS TBARS accumulation	- 0.8127	< 0.001

Table 4: Correlation between accumulation of carbonyl groups and antioxidant parameters in HV stage in patients with Eales' disease.

Variables (n=15)	r	P
Carbonyl Content Vs Plasma GSH	- 0.545	< 0.05
Carbonyl Content Vs SOD activity	- 0.391	< 0.05
SOD activity VS TBARS accumulation	- 0.764	< 0.001

increased TBARS accumulation in ED correlated with diminished SOD activity patients with ED (Tables.3 & 4).

Discussion

Protein peroxidation can occur by various mechanisms, (i) an increase in the production of ROS or (ii) decrease in the rate of scavenging of ROS, (iii) an increased susceptibility of the protein to oxidation and (iv) a decrease in the rate of removal of oxidized species (Jose et al., 1999). In this study we have found the impairment in SOD activity and diminished GSH content. Therefore this could be the possible reason for observing elevated protein carbonyl content in patients with ED. In our present study we have also found plasma GSH levels to be low in ED (Table 2).

The decrease in plasma GSH in ED may be an outcome of greater GSH consumption by ROS generated by phagocytes during inflammatory conditions, a phenomenon proposed by Thomas et al (1988). Our earlier work also revealed diminished GSH content in erythrocytes and vitreous of patients with ED (Bhooma et al., 1997 and Sulochana et al 1999). In addition to the above findings, Saxena et al (1999) and Srivatsava et al (2000) have reported reduced levels of platelet GSH and TBARS in patients with ED.

Lipid peroxides have been shown to condense with the side chain of protein amino acids resulting in the introduction of carbonyl groups in proteins (Refgaard et al., 2000). In the present study increased lipid peroxide accumulation was observed in patients with ED. Therefore as mentioned above increased accumulation of lipid peroxides can induce the

carbonyl groups in the proteins. This may be the other reason for increased protein carbonyl content observed in ED.

Carbonyl formation in proteins was found to be dependent on metal ions such as Fe^{2+} and Cu^{2+}. Fe^{2+} and Cu^{2+} can bind to the cation binding site in proteins and with help of H_2O_2 or O_2 they change the side chains of amino acids to carbonyl groups (Stadtman, 1990; Stadtman and Levine, 2000). Fe^{+2} and Cu^{+2} by involving in Fenton reaction, catalyze the production of hydroxyl radical, which have been shown to oxidize lipids, proteins and DNA (Moskovitz et al., 2002) These oxidation products have been shown to accumulate in various pathological conditions (Moskovitz et al.. 2002).

During intraocular inflammation, there would be disruption of blood-retinal barrier, this results in the invasion of circulating inflammatory cells and contact of serum components with retinal tissue, which trigger the expression of pro-inflammatory cytokines and augment respiratory burst in the phagocytes. These events result in the amplification of retinal inflammation and tissue damage (Lightman and Greenwood, 1992). Phagocytes have been shown to uptake the oxidatively modified proteins, and they have been reported to invoke immunological response which results in tissue damage during inflammatory condition (Merry et al., 1991).

In addition to the above, accumulation of carbonyl groups on protein results in series of chemical modifications, which ultimately results in the formation of advanced protein oxidation products (APO) or advanced glycation end products (AGE) (Miyata et al.,

2000). AGE on binding with its receptor on retinal microvascular cells (endothelial cells or pericytes) or retinal pigment epithelial cells, has been shown to induce the expression of cytokines, growth factors and proteases, which can result in retinal neovascularization (Stitt, 2001). Recent study by Swamy et al (2002) has revealed increased accumulation of AGE in plasma and ERM surgically excised from patients with ED. Therefore these could be the possible role/mechanism for oxidatively modified proteins in promoting retinal damage in ED.

Elevated levels of protein carbonyl groups content are reported in various diseases such as Alzheimer's disease (Smith et al 1998), Parkinson disease (Alam et al., 1997), diabetes mellitus (Baynes et al., 1999), rheumatoid arthritis (Renke et al., 2000), muscular dystrophy (Murphy and Kehrer, 1989), cataractogenesis (Garland et al., 1988), renal tumor (Uchida et al., 1995), uremia (Odetti et al., 1996), bronchopulmonary dysplasis (Gladstone and Levine, 1994) and amylotrophic lateral sclerosis (Bowling et al., 1993). Elevated carbonyl groups with diminished SOD activity and decreased GSH levels are seen in HV stage in ED. This suggests that oxidative stress still prevail as the disease progress to healing stages. This indicates that therapeutic measure through antioxidants might be helpful in preventing the damage inflicted by ROS to the retina in patients with ED. Recently, Rooji et al., (1999) have found that in patients with anterior uveitis, oral supplementation of vitamin C and E as adjuvant therapy improved the visual acuity. Also there are several reports for beneficial effects of antioxidant supplementation as adjuvant therapy in various diseases, where the role of free radical stress has been established (reviewed by McCall and Feri 1999).

CHAPTER 4

CHAPTER 4

8 – Hydroxyguanosine as the Biomarker for Oxidative DNA damage in Eales' Disease

Introduction

Free radials and other reactive species are constantly generated in vivo and cause damage to biomolecules, a process held in check only by the existence of multiple antioxidant and repair systems as well as the replacement of damaged lipids and proteins (Jose et al., 1999). DNA is probably the most biologically significant target of oxidative attack. ROS cause extensive DNA damage; including single-strand breaks and the formation of modified bases (Schöneich, 1999). One of the most abundant forms of oxidized base formed by the attack of $OH^{\bullet}$ on DNA is 8-OHdG. 8-OHdG has been unequivocally considered to be the early biomarker to assess the intensity of free radical-mediated tissue damage during metastasis, aging, neurodegenerative diseases, and inflammatory conditions (Dizdarogu, 1992).

Number of investigators have reported elevated 8-OHdG levels in patients suffering from various diseases such as chronic hepatitis (Shimoda et al., 1994), Fanconi's anemia (Takeuchi et al., 1993), diabetes mellitus (Rehman and Halliwell, 1999) and *Helicobacter pylori* infections (Baik et al., 1996). However there are no human clinical reports available on the levels of 8-OHdG in ocular inflammatory disease including ED. Hence in the present study, the levels of 8-OHdG in leukocytes from ED were determined.

Results

Standardization for determination of 8-OHdG by GC-MS

Fig.1 depicts the mass spectrum of authentic standards of 8-OHdG and 2'deoxyguanosine (2dG). 8-OHdG had the retention time of 31.05 min, while 2dG had retention time of 31.6 min respectively. Fig. 2 represents the selective ion monitoring (SIM) analysis of 8-OHdG. Fig. 3 represents the selective ion monitoring (SIM) analysis for 2dG. Fig. 4 depicts typical mass spectrum of test sample, while Fig. 5 shows the mass spectrum of control sample.

8-OHdG and antioxidant status in ED.

Table 1 describes the levels of 8-OHdG content along with other antioxidant/oxidant parameters studied in patients with ED and control subjects. Levels of 8-OHdG was 3.3 and 1.8 fold, higher in AV stage and HV stages respectively in patients with ED, when compared with healthy control subjects (Tables. 1 & 2). Increased accumulation of 8-OHdG in AV and HV stages of ED correlated with diminished SOD activity and plasma GSH content (Tables. 3 & 4).

Plasma GSH content was decreased by 2.3 and 1.6 fold respectively in AV and HV stages of ED, when compared with healthy control subjects (Tables. 1 & 2). SOD activity was diminished by 1.8 and 1.3 fold respectively in AV and HV stages of ED, when compared with control subjects (Tables. 1 & 2). TBARS accumulation was 1.6 and 1.2 fold higher respectively in AV and HV stages of ED when compared with healthy control subjects

Table 1: Levels of leukocyte 8-OHdG , plasma GSH , and erythrocyte TBARS content and SOD activity in patients with Eales' disease and healthy control subjects.

Group	8 OHdG (residue/10^6 dG)	Plasma GSH (μM)	SOD (Unit/g Hb)	TBARS (nmole MDA/g Hb)
Active vasculities (n=12)	10.2 ± 1.8	1.61 ± 0.15	2536 ± 503	41.82 ± 2.66
Heated vasculities (n=10)	5.8 ± 0.14	2.2 ± 0.31	3170 ± 245	32.31 ± 3.51
Healthy Control Subjects (n=14)	3.02 ± 0.38	3.1 ± 0.15	4412 ± 138	26.11 ± 4.12

All values are expressed as Mean ± SD.

Age / sex

Active Vasculitis: 26.33 ± 4.2 years, all Males.

Healed Vasculitis : 24.71 ± 4.75 years , all Males.

Healthy Control subjects : 27.78 ± 2.23 years, all Males.

Table 2: Schefee' post hoc test for multiple comparison

Comparison	8-OHdG/10^6 dG	GSH (μM)	SOD (Unit/g Hb)	TBARS (nmole MDA/g Hb)
Active Perivasculities*	P < 0.001	P < 0.001	P < 0.001	P< 0.001
Healed perivasculities*	P < 0.001	P < 0.001	P <0.001	P < 0.001
Active perivasculities vs Healed perivasculities	P < 0.001	P < 0.001	P < 0.05	P < 0.05

***Comparison with respect to healthy controls subjects.**

Table 3: Correlation between accumulation of 8-OHdG and antioxidant parameters in patients with ED in AV stage.

Variables (n = 12)	r	P
8-OHdG Vs SOD	- 0.8068	< 0.001
8-OHdG Vs GSH	- 0.9771	< 0.001
SOD Vs TBARS	- 0.9142	< 0.001

Table 4: Correlation between accumulation of 8-OHdG and antioxidant parameters in patients with ED in HV stage.

Variables (n =10)	r	P
8-OHdG Vs SOD	- 0.763	< 0.001
8-OHdG Vs GSH	- 0.917	< 0.001
SOD Vs TBARS	- 0.670	< 0.05

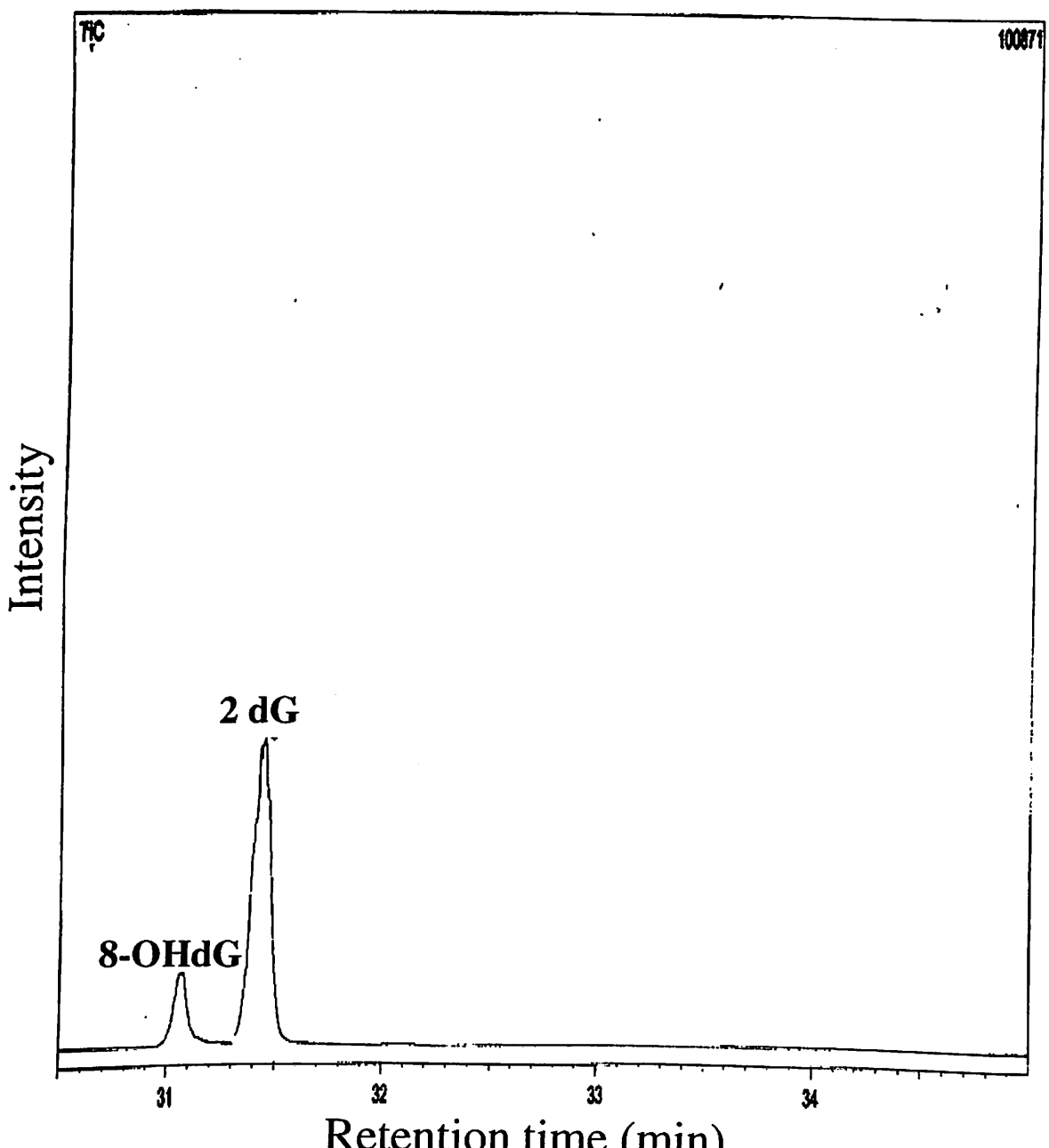


Figure.1: Ion current profile of authentic 8-OHdG and 2dG standards

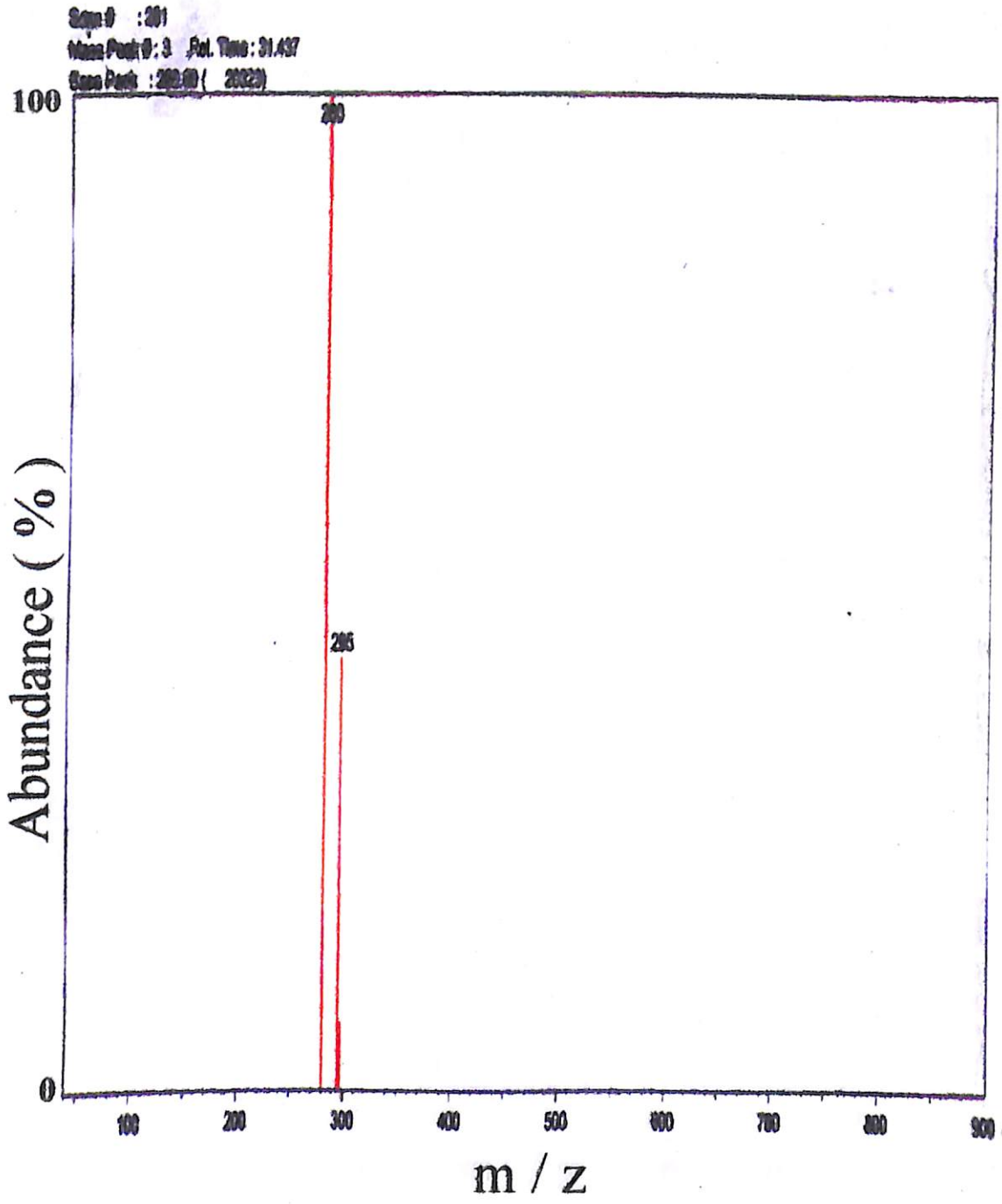


Figure.2 : Mass spectra of 2dG in SIM mode.

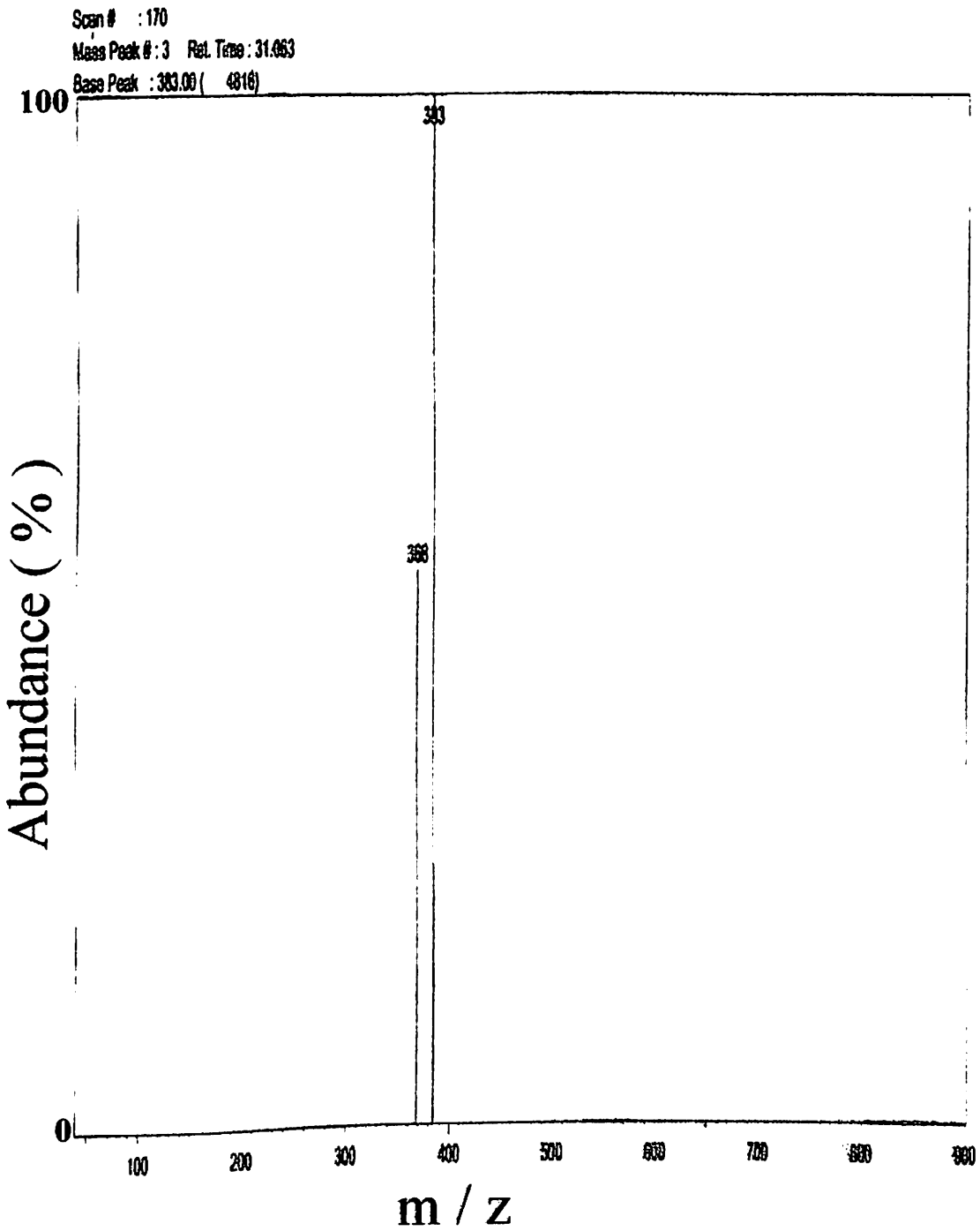
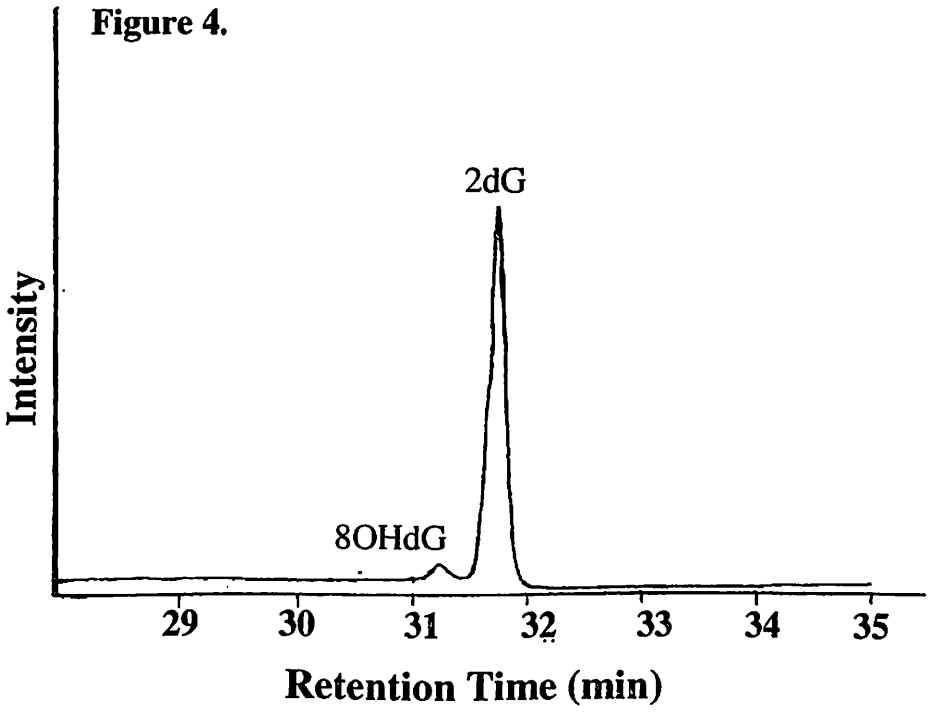


Figure.3 : Mass spectra of 8-OHdG in SIM mode.



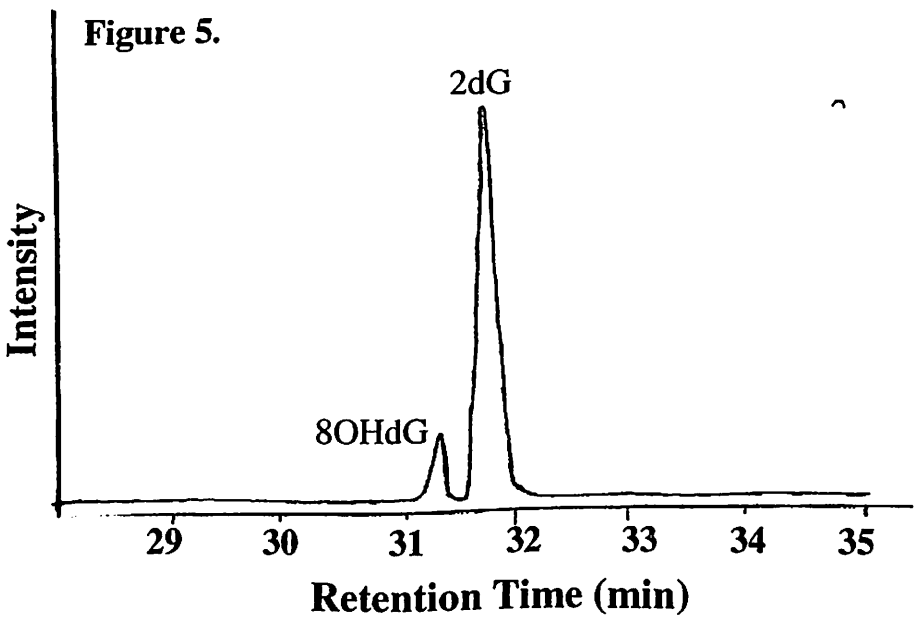


Figure 4: Ion current profile of 8OHdG and 2dG in control sample.
Figure 5: Ion current profile of 8OHdG and 2dG in Eales' disease sample

(Tables. 1 & 2). Increased TBARS accumulation in ED correlated with diminished SOD activity patients with ED (Tables. 3 & 4).

Discussion

DNA oxidation can occur by various mechanisms, (i) an increase in the production of ROS or (ii) decrease in the rate of scavenging of ROS, (iii) an increased susceptibility of the DNA to oxidation and (iv) a decrease in the rate of removal of oxidized bases (Wiseman and Halliwell, 1996). 8-OHdG is the most abundant form of DNA adducts which is formed by direct attack of DNA by $OH^{\bullet}$ (Kasai et al, 1984). Another reason for considering the determination of 8-OHdG is that it is considered to be the early marker to assess the intensity of free radical damage to the tissues (Takeuchi et al., 1994).

During inflammatory conditions, activation of leukocyte results in increased respiratory burst, which is associated with increased production of ROS. Therefore if unchecked, ROS produced will damage the biomolecules, which results in the loss of their structure and function (Aust and Eveleigh, 1999). Inove and Kawanishi (1995) have reported the increased formation of 8-OHdG in leukocytes when stimulated with bacterial lipopolysaccharide (LPS), they attribute this to simultaneous generation of NO and $O_2^{\bullet-}$. The decrease in plasma GSH in ED may be an outcome of greater GSH consumption by ROS generated by phagocytes during inflammatory conditions (Thomas et al., 1988).

The formation of endogenous DNA damage in cells involves Fenton reaction. DNA damaging Fenton reaction must occur in close proximity to DNA because, $OH^{\bullet}$ react

readily with nearly all biological compounds and thus do not diffuse very far from their site of generation (Kawanishi et al., 2001 ; Filho and Meneghini, 1991). Therefore, the formation of oxidative DNA damage in cells is expected to depend on the rate of production of free radicals and oxidants, and the ability of iron to diffuse in to DNA (Kawanishi et al., 2001 ; Filho and Meneghini, 1991). Additionally, free radicals and oxidants induce the release of metal ions from proteins, which in turn generate more powerful oxidants, which in turn promotes DNA damage (Imlay et al., 1988). As mentioned earlier during intraocular inflammatory conditions, there would be disruption of blood-retinal barrier and this result in the infiltration of circulating inflammatory cells in to the retina (Lightman and Greenwood 1992). Once these inflammatory cells are triggered by inflammatory stimuli, they undergo respiratory burst resulting in production of H_2O_2 (Root and Metcalf, 1977). The H_2O_2 produced directly oxidizes the proteins present intracellularly to release iron and copper.

The release of transition elements from proteins catalyses Fenton reaction, resulting in the production of $OH^{\bullet}$ (Fantone and Ward, 1982). $OH^{\bullet}$ thus produced can attack DNA, resulting in the formation of 8-OHdG. On the other hand, H_2O_2 can directly induce the gene expression of proinflammatory cytokines such as interleukin 1 (IL-1), tumor necrosis factor α (TNF -α). These cytokines stimulate the inflammatory cells in autocrine fashion, which aids in clonal proliferation of the inflammatory cells, enhancing the production of ROS (Guy et al., 1989). These events could eventually amplify the inflammatory cascade and mediate free radical meditated retinal tissue damage.

Accumulation of 8-OHdG has been shown in several diseases [reviewed by Griffiths et al., 2002]. Shin et al., (2001) have demonstrated increased serum levels of 8-OHdG in patients non insulin dependent diabetes mellitus (NIDDM), with or without various micro and macrovascular complications. However they have not simultaneously determined the antioxidant parameters in their study. In the present study simultaneous determination of both antioxidant status and accumulation of 8-OHdG has been performed. Gackowski et al., (2001) and Sentürker et al., (1997) have reported increased 8-OHdG accumulation which correlated with the elevated levels of oxidants and diminished antioxidant enzymes in their leukocytes as well as in plasma of patients with artherosclerosis and myeloid leukemia respectively.

Lee et al., (1998) have demonstrated that supplementation of antioxidants 200 IU vitamin E, 9 mg of β carotene, 500 mg of vitamin C or 1.8 g of red ginseng per day for four weeks to chronic smokers decreased the levels of leukocyte 8-OHdG and plasma protein carbonyl content. Similarly, Djuric et al., (2001) have reported that supplementation of 50 mg soy /day isoflavones for three weeks to smokers, deceased the levels of 8-OHdG. In addition several antioxidant supplementation intervention studies have revealed diminished levels of 8-OHdG in leukocytes of human subjects. In the present study, 8-OHdG has been found to be an apt marker for oxidative DNA damage and the results are consistent with the reports of other investigators. Hence it is believed that antioxidant vitamin C and E supplementation might be beneficial to patients with Eales' disease.

CHAPTER 5

CHAPTER 5

Identification, Purification and Characterization of Novel 88 kDa protein from Serum and Vitreous of patients with Eales' Disease

Introduction

Serum, derived from plasma with clotting factors removed, contains about 60 – 80 mg protein / ml in addition to various small molecules including salts, lipids, amino acids and sugars. The major protein constituents of serum include albumin, immunoglobulins, transferrin, haptoglobin and lipoproteins. In addition to these major constituents, serum also contains many other proteins that are synthesized and secreted, shed or lost from cells and tissues throughout the body. It is estimated that up to 10,000 proteins may be present commonly in serum, most of which would be present at very low relative abundance. During pathological conditions, there would be destruction of cells, therefore the contents of cells are extruded and released into the circulation. The presence of these protein components in blood reinforces the benefit of using them as biomarker for disease states or can be used to understand the disease process. Identification of human plasma serum proteome has led to the development of several diagnostic markers for different types of malignancies, autoimmune diseases such as rheumatoid arthritis.

Since the etiology of ED is considered to be idiopathic, identifying the novel proteins expressed in serum or other biological specimens would shower insights into pathomechanism of ED. Hence efforts were taken to identify, purify and characterize any novel protein in serum and vitreous of patients with ED. This chapter describes about the

identification, development of purification techniques and characterization of novel 88 kDa protein found in serum and vitreous of patients with ED.

RESULTS

Purification of 88 kDa protein

A new protein has been identified in serum and vitreous of patients with ED. In 7.5 % NPAGE, it resolved in β_1 glycoprotein region (Fig. 1). The presence of new protein in the fractions was analyzed by performing 7.5 % NPAGE followed by silver staining. The elution profile of the proteins that were eluted from preparative PAGE system is depicted in Fig.2. Initial experiments revealed that the new protein exhibited anti TBARS activity *in vitro* when incubated with RBC. Hence this was used as the marker to identity the protein in subsequent purification steps. The new protein eluted between 150-175 fraction. All these fractions were pooled and concentrated using lyophylization. Then further purification was performed using RP-HPLC. In the RP HPLC the protein had a retention time of 10.2 min (Fig.3). The steps involved in the purification and the yield of protein from serum and vitreous are given in Table 1.

Physiochemical properties of the 88 kDa protein

Molecular weight

Molecular weight (MW) of the new protein was determined using RP HPLC – gel permeation chromatography. New protein was found to have the apparent MW of 88 kDa and hence it was designated as 88 kDa protein (Fig.4). SDS-PAGE also revealed similar results (Fig.5). However, the exact oligomeric nature of 88kDa protein is not known at the present.

Figure 3

HPLC elution profile showing the homogeneity of the 88kDa protein from (A) serum and (B) vitreous (5 µg each) with retention time of 10.2 min. The separation was performed using RP C18 column 5 µm particle size with 300 A° pore size (Lichrosphere, Merck, Germany).

Figure 4

Molecular weight determination of the purified protein. This was done using gel filtration column (TSK 3000, Amersham Pharmacia, Sweden) using LKB Bromma HPLC system.. The gel permeation molecular weight markers used were urease dimer (545 kDa), urease monomer (272 kDa), BSA dimer (132 kDa), BSA monomer (66 kDa) and carbonic anhydrase (49 kDa). B: 7.5% native PAGE of purified protein stained by silver nitrate indicating its homogeneity. Lane 1, Serum (20 µg) from patient with Eales' disease, Lane 2, purified 88 kDa protein (30 µg).

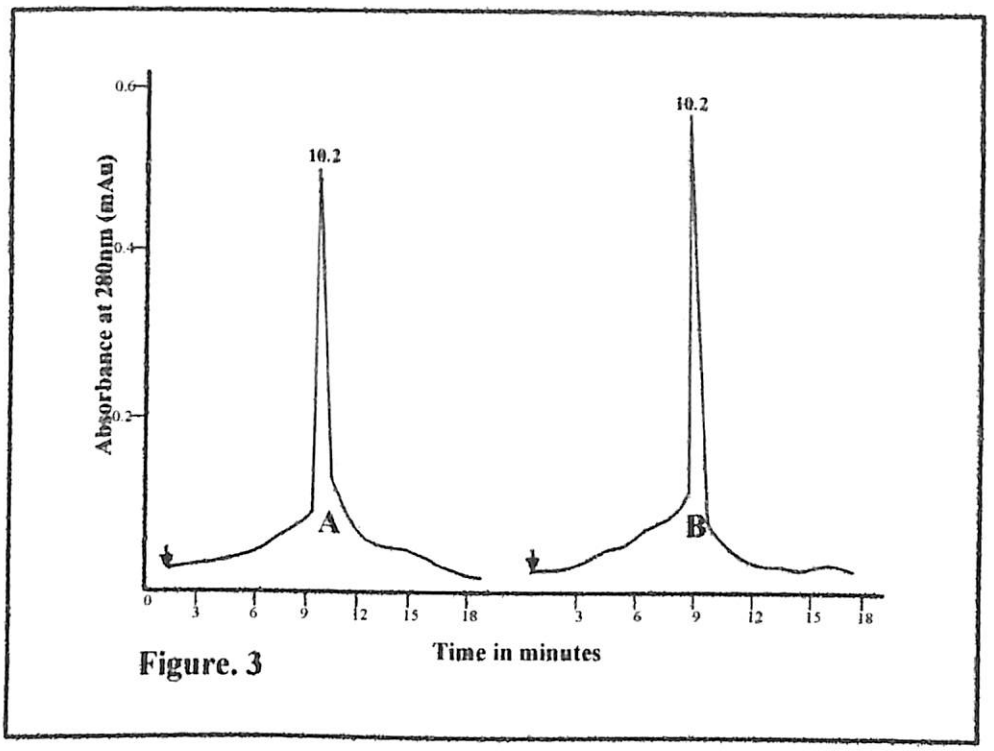


Figure. 3

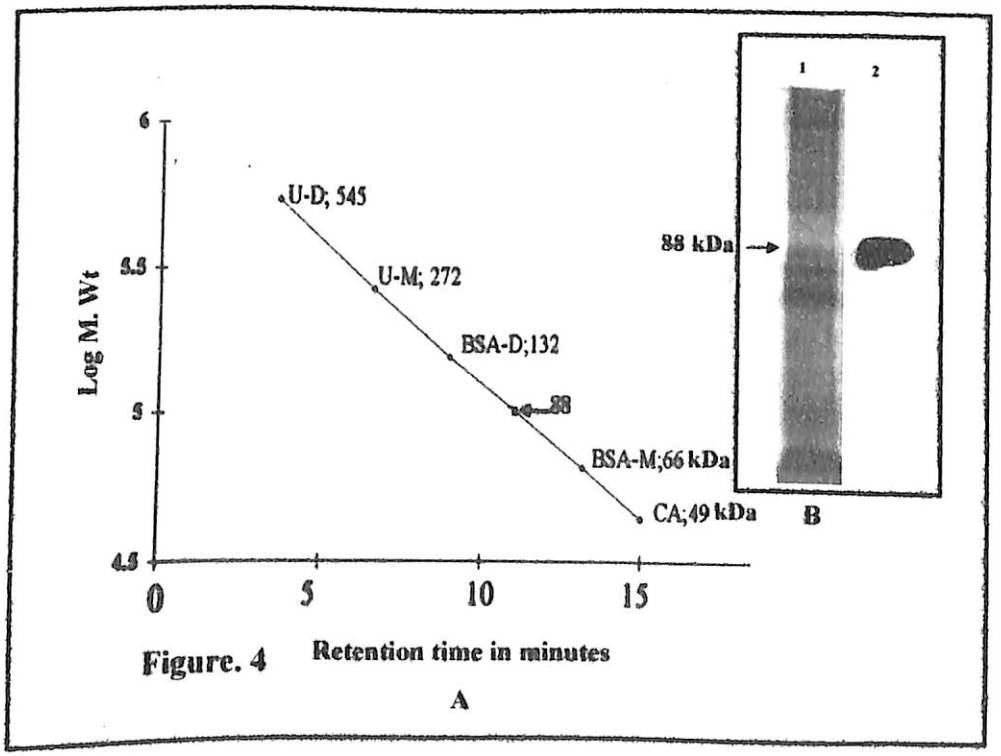


Figure. 4

A

Thermal stability of the 88 kDa protein

0.5 µg 88 kDa protein /100 µl PBS pH 7.4 was incubated at various temperatures starting from 37°C - 70°C for varying time points (10 min – 60 min) and then anti TBARS activity was determined in these tubes. Results showed that 88 kDa protein was completely destroyed at 70°C when heated for more then 20 min (Table 2).

pI of 88 kDa protein

pI determination of the purified 88 kDa revealed the pI at pH 5.0

88kDa protein is a glycoprotein

Purified 88 kDa protein along with standard glycoprotein (ovalbumin) were resolved in SDS PAGE and PAS stain was performed to identify whether 88 kDa protein is glycosylated the positive pink staining indicated that 88 kDa is a glycoprotein (Fig. 6)

N terminal and internal peptide sequence of 88 kDa

The N terminal sequence of the protein is given in Table 3. When the protein was enzymatically hydrolyzed using trypsin it yielded several peptides varying in optical density (OD at 214 nm in RP HPLC- UV detection). Three major peptides were selected based on OD at 214 nm, and subjected to amino acid sequencing. The sequences are given in Table 4. The sequence homology search on database revealed no match to the sequences of the 88 kDa protein. Hence 88 kDa protein was considered as a novel protein. In physical and biochemical properties 88 kDa protein resembled certain other

Table 2: Thermal denaturation studies on 88 kDa protein

Sl. No.	Temperature	Time	anti TBARS activity (%)
I	37° C	10	97 ± 2
		20	97 ± 2
		30	97 ± 2
		40	97 ± 2
		50	97 ± 2
		60	97 ± 2
II	42° C	10	94 ± 1.6
		20	94 ± 1.6
		30	93 ± 2.9
		40	91 ± 0.8
		50	90 ± 0.5
		60	90 ± 0.6
III	50° C	10	89 ± 1.1
		20	86 ± 0.9
		30	84 ± 1.2
		40	82 ± 1.8
		50	82 ± 0.3
		60	79 ± 1.1
IV	60° C	10	74 ± 1.6
		20	72 ± 0.2
		30	69 ± 0.8
		40	65 ± 1.5
		50	61 ± 0.6
		60	51 ± 0.9
V	70° C	10	35 ± 2.9
		20	20 ± 4.3
		30	9 ± 3.1
		40	ND
		50	ND
		60	ND

The results were average of 3 independent experiments.

Table 3: Sequence of 28 amino acids from N-terminus of 88 kDa protein

1															28
A	D	D	P	N	S	L	S	P	S	A	F	A	E	A	L	A
L	L	R	D	S	X	L	A	R	F	V					

Conventional one letter symbols are used for amino acids

X - not determined

Table 4: Internal peptide sequence derived from tryptic digestion of purified 88 kDa protein

Peak No.	Retention Time	Sequence
12	18	SKVHDSYN
14	21	SSGTSYAALA
17	23	SPSYEFEEDE

Figure 5

12.5 % SDS PAGE gel profile for purified protein. Lane 1: molecular weight markers, Lane 2: purified protein from either serum or vitreous. The presence of homogeneous protein ~ 88 kDa was observed.

Figure 6

PAS stain for detecting glycoprotein. 12.5 % SDS PAGE gel profile, indicating the glycosylation of 88 kDa protein. Lane 1: Molecular weight markers, Lane 2: purified protein from vitreous of patients with ED, Lane 3: purified protein from serum of patients with ED. Lane 4: human albumin (monomer) was used as positive control.

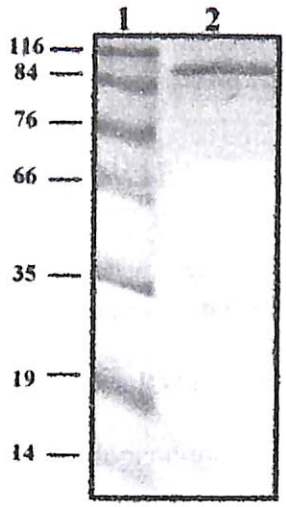


Figure. 5

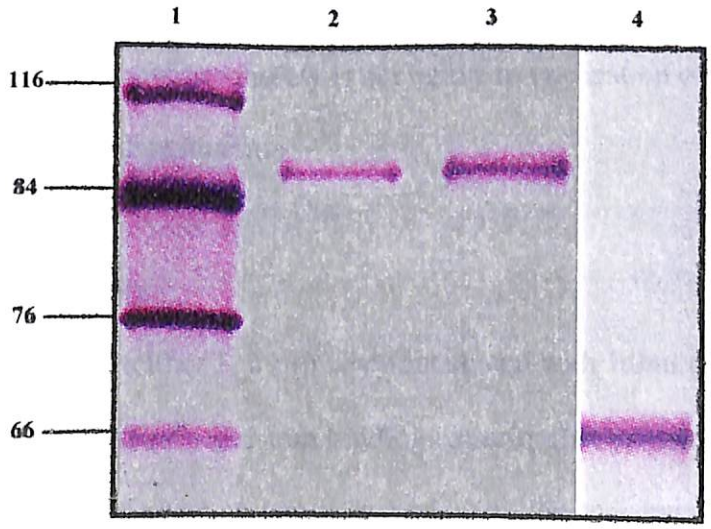


Figure. 6

acute phase reactants. The common properties and the differences between 88 kDa protein and other similar proteins are described in Table 5.

Biochemical Characteristics of 88 kDa protein

Anti TBARS activity

88kDa protein had anti TBARS activity in the *in vitro* assays. This property was used routinely for its identification besides PAGE electrophoresis during purification steps. The anti-TBARS activity was dose dependent on protein concentration. It was tested for this effect on erythrocytes of patients with diabetes and without diabetes and also patients with Eales' disease (Table 6). Both serum and vitreous derived 88kDa protein showed similar extent of anti TBARS activity. The conventional thiol group inhibitors namely DTNB and PCMB inhibited this activity of the protein, indicating the importance of thiol residues antiTBARS actitity of 88kDa protein (Table 7). This was further confirmed by the fact that total thiols contributed to 0.8 % of total mass of the protein. The *in vitro* demonstration of anti TBARS activity in neutral pH is suggestive of the fact that the contribution by carbohydrate moiety is negligible as enolisation of sugar (in alkaline pH) is needed for antioxidant function.

Iron binding capacity

The purified 88kDa (either 1, 2 μg) when incubated with 1.0ml of human serum before estimating free iron (Fe^{2+}) and iron binding capacity (IBC) found to decrease the level of iron and IBC. This effect was dose dependent on the concentrations of 88kDa protein used. The results are summarized in (Table 8).

Table 5: Comparative table of 88 kDa protein with other similar proteins

Protein	Common property	Difference from 88 kDa protein
Transferrin	Binding with iron Acute phase protein	Molecular weight (77 kDa) Not a glycoprotein No homology in N terminus
Haptoglobin	Iron binding, Hemoglobin binding Agglutination, Electrophoretic movement (β_1 region) raised in inflammation, mol.wt 90 kDa.	Seen in all individuals irrespective of their disease. No sequence homology with 88 kDa protein in N terminus
Ceruloplasmin	Iron binding, acute phase protein feroxidase activity, endogenous antioxidant.	Not having ceruloplasmin activity with paraphenylene diamine, No sequence homology in N terminus
Superoxide Dismutase	Endogenous antioxidant	But did not inhibit epinephrine auto oxidation (No SOD activity)
Glutathione Peroxidase (GP_x)	Removes H_2O_2 .	88 kDa does not require GSH for activity, while GP_x needs GSH for its activity.

Table 6: Effect of purified 88 kDa protein on erythrocyte TBARS levels

Condition	TBARS levels of RBCs alone (nm MDA dL^{-1})*	TBARS levels of RBC in the presence of 88 kDa protein (nmMDAdL^{-1})*		% decrease in erythrocytes TBARS activity in the presence of 88 kDa protein	
		+ 100 ng of protein	+ 200 ng of protein	100 ng of protein	200 ng of protein
Eales' (n =10)[1]	2930 ± 282	2154 ± 154	1874 ± 131	25.6**	48**
Diabetes (n =10)[2]	566 ± 28	449 ± 19	372 ± 19	20.7**	34**
Normal control (n =10)[3]	405±33	344 ± 25	286 ± 21	15**	30**

* The values are in mean ± SD, ** $P < 0.05$, when compared with RBCs alone.

Age/sex
1. 20-40 years in Eales' group (all males)
2. 40-60 years in diabetes (8 men, 2 females)
3. 15-45 years in Control group (6 men, 4 females)

Table 7: Effect of thiol inhibitors on the anti TBARS activity of 88 kDa protein

Inhibitors	Concentration μM	Anti TBARS activity Relative activity (%)
None	----	98 ± 2
DTNB	0.05	25 ± 2 **
	0.10	ND **
PCMB	0.05	37 ± 5.2 **
	0.10	8 ± 4 **

The results were average of three independent experiments. ND – not detected. ** $P < 0.001$ when compared with experiments without the addition of thiol inhibitors

Table 8: Effect of 88 kDa protein on serum iron and total iron binding capacity

Serum constituents (μgm/dL)	No 88 kDa protein	Purified 88 kDa protein	
		1 μg /1 ml serum	2 μg / 1 ml serum
Fe^{2+}	193 ± 22	100 ± 16**	82 ± 1 3**
Total iron binding capacity (as Fe^{2+})	484 ± 36	286 ± 28**	214 ± 20**

The values are expressed as mean ± SD of five individual experiments. All the samples were from normal volunteers (two males and three females) of age group 25 to 40 years. ** $P < 0.05$, when compared with serum without addition of 88 kDa protein.

Figure 7

Immunoelectrophoresis of serum 88kDa protein with Eales' disease. (A) Well 1 serum (60 µg) from patient with Eales' disease, well 2 vitreous (10 µg) from patient with Eales' disease. (B) Purified 88kDa protein from vitreous (2 µg, well 1) and serum (2 µg, well 2) from patients with Eales' disease.(C) Well 1 vitreous (60 µg of protein) from a patient with Eales' disease, well 2 vitreous (10 µg of protein) from patient with diabetic retinopathy. (D) Well 1 serum (60 µg of protein) from patient with Eales' disease, well 2 serum (10 µg of protein) obtained from normal healthy volunteer. In all the above experiments (Fig 4A-D), the central well was probed with anti 88kDa antibody (goat polyclonal 1:500 dilution.

Figure 8

Western blot analysis of purified 88kDa protein. Lane 1, 30 µg purified 88kDa from serum. Lane 2, 30 µg purified 88kda from vitreous.

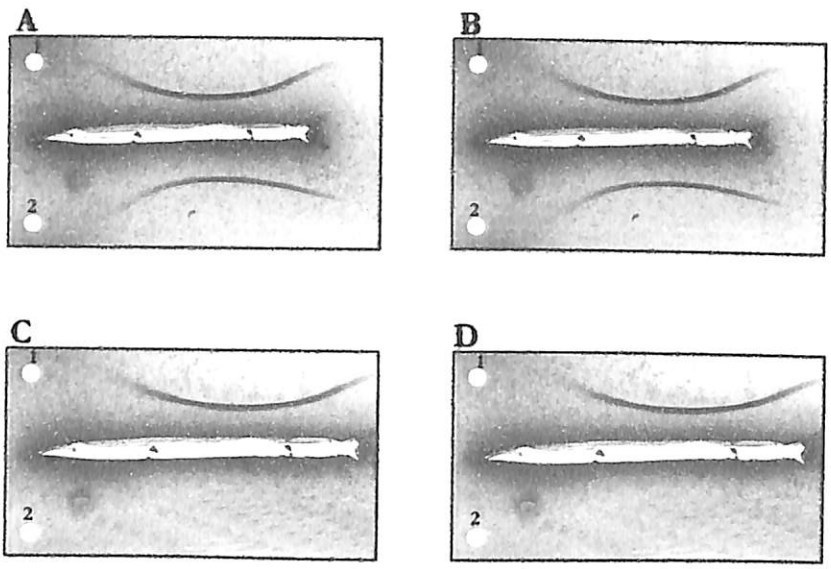


Figure . 7

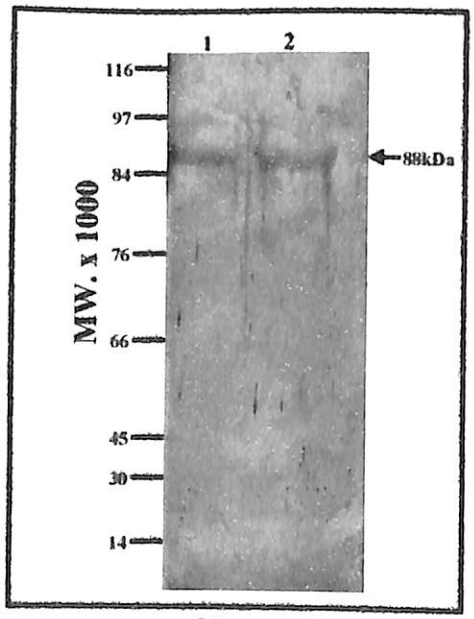


Figure. 8

Figure 1.

7.5% native PAGE, stained by Commassie brilliant blue G250. Lane 1, and 7 serum from normal healthy volunteer, Lane 2,5 and 6 are serum from patients with Bales' disease, Lane 3 and 4 serum from patients with diabetic retinopathy. The presence of 88kDa protein in Lanes 2,5 and 6 is indicated by arrow in the photomicrograph. 20 µg of serum protein was uniformly used for protein profile.

Figure 2

Elution profile of proteins from preparative cell electrophoresis system. 88 kDa protein gets eluted from 150 -175 fractions, which is represented by dashed line in the graph. Anti TBARS activity was maximum in these fractions.

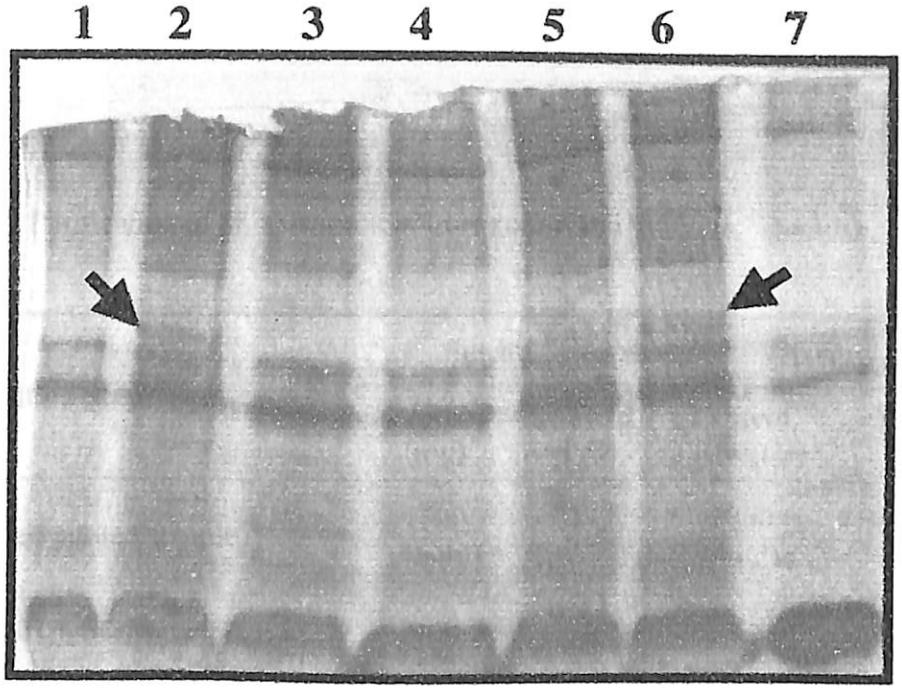


Figure. 1

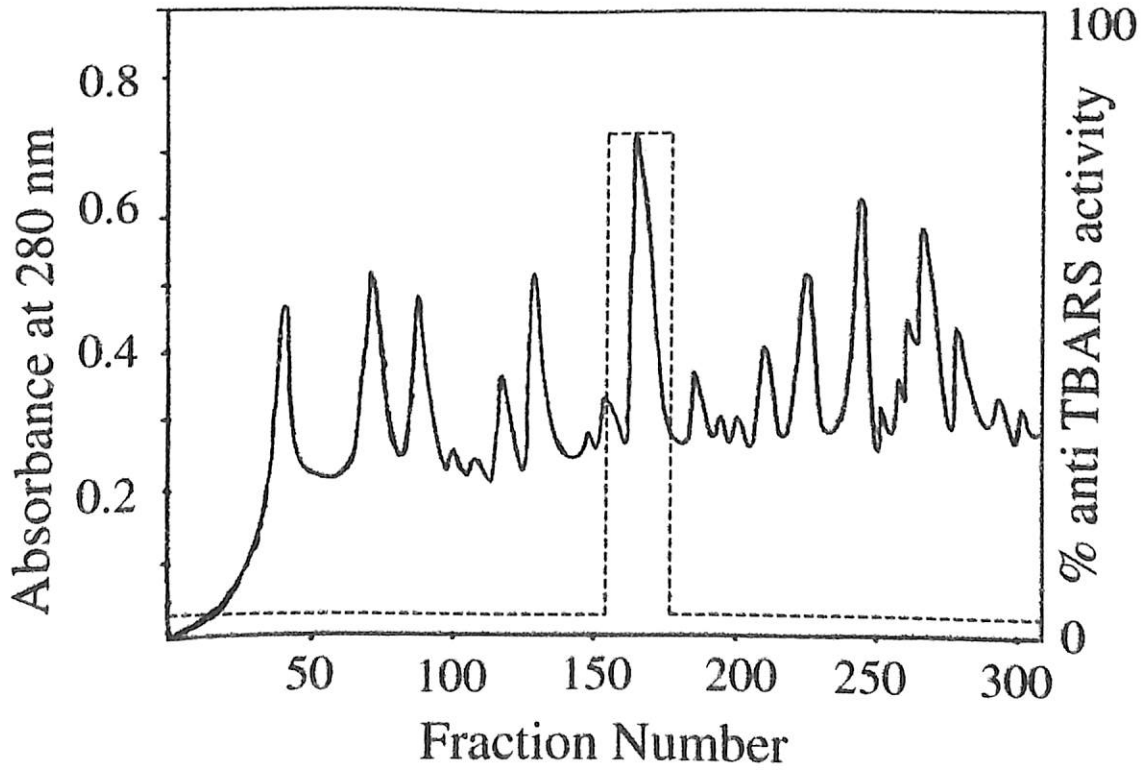


Figure. 2

Table 1: Purification of 88 kDa protein from serum and vitreous.

Purification Step	Serum		Vitreous	
	Protein (mg)	Yield %	Protein (µg)	Yield %
Undiluted pooled sample	1500 (15 ml)	100	2000 (4 ml)	100
60 % $(NH_4)_2 SO_4$ precipitation	700	46	--	--
5 % preparative gel electrophoresis	50	3.3	100	5
RP- HPLC	1.5	0.1	20	1

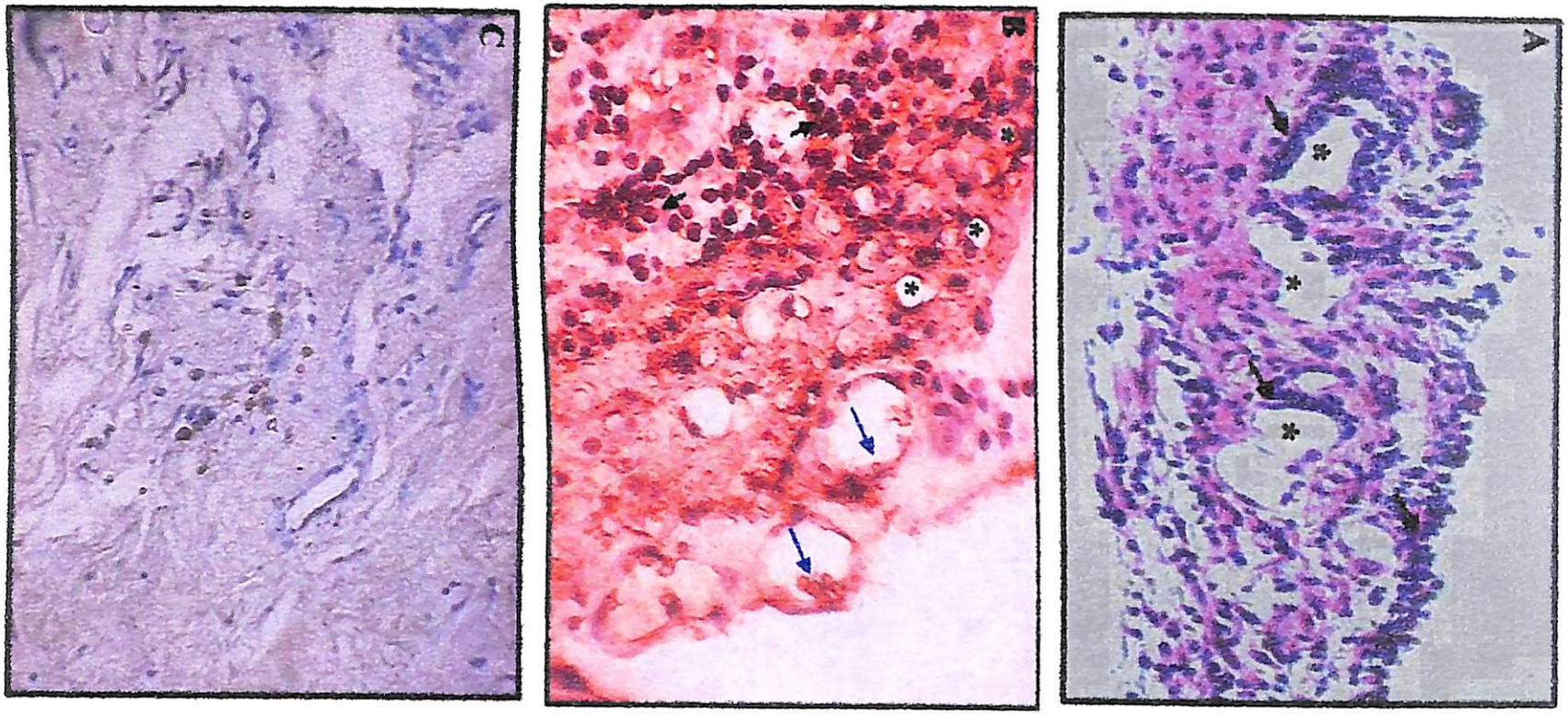
Table 1: Purification of 88 kDa protein from serum and vitreous.

Purification Step	Serum		Vitreous	
	Protein (mg)	**Yield %**	**Protein (µg)**	**Yield %**
Undiluted pooled sample	1500 (15 ml)	100	2000 (4 ml)	100
60 % $(NH_4)_2 SO_4$ precipitation	700	46	--	--
5 % preparative gel electrophoresis	50	3.3	100	5
RP- HPLC	1.5	0.1	20	1

Figure 9

Immunohistochemical localization of 88 kDa protein in ERM of patients with Eales disease. (A) Haematoxylin and eosin staining of ERM obtained from patients with Eales disease, showing neovascular channels with dense leukocyte infiltration. Neovascular channels are indicated by black asterisks and the infiltrating leukocytes in the neovascular endothelium are represented by black arrows, X 200 magnification. (B) Immunolocalization for 88 kDa protein in ERM in Eales' disease. The expression of 88 kDa protein in infiltrating leukocytes are indicated by solid black arrow, while in the neovascular channels endothelium it is denoted by solid blue arrow, while the immunoreactivity of vascular endothelium for 88kDa protein is represented by black asterisks, X 400 magnification. (C) ERM obtained from patients with diabetic retinopathy did not reveal 88 kDa protein expression. X 200 magnification.

Figure. 9



Haptoglobin like activity for 88 kDa protein

Purified 88 kDa protein exhibited agglutination activity, suggesting the possible haptoglobin activity. The common properties and the differences between 88kDa protein and other similar proteins are given as a comparative chart in Table 5.

Specificity of anti 88 kDa antibody

To evaluate the specificity of anti 88 kDa antibody, immunoelectrophoresis and Western blot analysis were performed. Results of these experiments are illustrated in (Fig. 7 and 8). Western analysis showed that the purified 88kDa protein from serum and vitreous were homogeneous. Immunoelectrophoresis revealed that 88kDa protein from serum and vitreous were immunologically identical. In addition to this western analysis of serum and vitreous samples from patients with diabetic retinopathy or normal healthy volunteer (only serum) revealed the absence of 88 kDa protein indicating the specificity of the polyclonal antibody produced against 88kDa protein.

Immunolocalization of 88 kDa in ERM of ED

Fig 9 A illustrates the Hematoxylin and Eosin staining of ERM of ED patients, showing neovascular channels with infiltration of leukocytes. Immunoreactivity for anti 88 kDa antibody was detected in infiltrating leukocytes, neovascular channels and in the endothelium (Fig. 9 B) in all the ERM studied (n = 4). On the other hand, ERM from patients with diabetic retinopathy (n = 8) did not reveal the presence of 88 kDa protein expression (Fig. 9 C).

Discussion

Most tissues are protected from iron and haem-mediated oxidative damage by two mechanisms. Either ROS are scavenged rapidly by enzymes such as SOD, catalase and GPx or the generation of ROS is prevented by sequestering iron or haem extracellularly as protein complexes (Guttridde and Halliwell 1988; Halliwell and Guttridge 1989).

When haem is released as a result of trauma, cellular necrosis, hemolysis or the turnover of haem proteins, it is rapidly bound by hemopexin and delivered principally to the liver where it is internalized (Smith and Morgen 1978 & 1994) and catabolized (Smith, 1990). In many cells including retinal pigment epithelial (RPE) cells binding of heme-hemopexin sets in motion for series of protective biochemical events (Hunt et al., 1996). Hemopexin also protects against peroxidative damage due to hemoglobin by binding heme released from methemoglobin or from oxyhemoglobin in the presence of hydrogen peroxide which is likely to be present at the site of inflammation (Miller et al 1996).

However, hemoglobin can act as peroxidase upon release from lysed RBCs is bound to haptoglobin, (Everse et al., 1994. The levels of hemopexin and haptoglobin are found to be increased in conditions of inflammation (Reynes et al., 1991; Chancellor et al., 1995) or during hypoxia (Wenger et al., 1995).

The retina is especially vulnerable to attack by ROS. It contains high levels of polyunsaturated fatty acids (Battaglia and Albert, 1989) which can undergo peroxidation; because it is the highly oxygenated tissue containing abundant mitochondria

(Adler,1992). Moreover unlike most tissues, the retina is subject to light irradiation; this contributes to increased levels of ROS (Rozanowska et al., 1995). Finally, proteins of outer segments of photoreceptors are shed on a diurnal basis (Bok., 1993) and internalized by retinal pigment epithelial cells (RPE) cells leading to H_2O_2 production (Miceli et al., 1994 ; Dorery et al., 1989). Therefore optimum levels of antioxidants are necessary to protect the retina against oxidative damage.

Formation of ROS requires redox-active transition metals such as iron and copper. (Guttridge and Halliwell, 1988). Haem and certain haem-proteins can also accept and release electrons (Vincent et al., 1988). Free haem and iron may be released in to the retina from haem/iron-containing proteins upon cellular necrosis and injury, while hemoglobin might enter as the result of hemorrhage. In addition, iron may be present in the retina during the transport across the barrier cells from the circulation to the neural retina (Hunt and Davis, 1992). Since the retina is cutoff from the circulation by the blood-retinal barrier (BRB) (Peyman and Bok, 1972). Liver synthesized protective proteins for e.g. hemopexin, haptoglobin cannot enter the neural retina unless there is disruption of BRB.

In ED, disruption of BRB takes place due to due to intra-ocular inflammation (Lightman and Greenwood, 1992). This could result in seepage of blood into the retina. Therefore it is predicted that to protect the retina against oxidative stress. The fact that elevated levels of 88kDa protein in vitreous than in serum have been observed, indicates that 88kDa protein in circulation might have reached the retina due to the breakdown of the blood

retinal barrier during inflammation or this might have been produced locally. Morevoer, vitreous is considered to be the molecular repository for retinal metabolism (Reddy, 1979). 88 kDa protein resembles in function to that of transferrin and haptoglobin, tough there was no homology with N terminal sequence and internal peptide amino acid sequences with that of the above-mentioned proteins. In addition to the above, 88 kDa protein found in serum and vitreous were found to be immunologically identical (Fig. 7).

Wu et al., (1996) has reported the expression of a novel 76 kDa protein in RPE. Partial sequencing of this protein revealed that it belonged to the class of transferrin. Further studies on this protein, revealed its ability to quench superoxide in cultured retinal pigment epithelial cells (RPE). In addition to the above, elaboration of this protein has been shown in experimental autoimmune uveitis (EAU), suggesting the acute phase response of this protein. Similarly 88 kDa protein has been predicted to be one of the APR proteins expressed in ED. 88 kDa acts as an antioxidant by its ability to inhibit TBARS formation. This is mainly due to the thiol groups in the protein, which acts as electron sinks, whereby it scavenges free radicals. This was confirmed by the fact that the total thiol content of 88 kDa protein was found to be 0.8 % of the total mass of the protein. Anti TBARS activity of 88 kDa protein was inhibited when incubated with thiol inhibitors such as DTNB and PCMB, indicating vital role of thiol groups for antioxidant activity of 88 kDa protein. 88 kDa protein also has the ability to sequester iron, thus it can inhibit the potentially hazardous Fenton reaction.

Since N terminal amino acid and internal peptide sequence of 88 kDa did not give suitable hits with any of the protein described in database, this protein was considered as novel. Therefore it is presumed that, 88kDa protein might be expressed to protect the retina form oxidative stress. However, cloning of 88 kDa protein could help in deciphering its exact identity, molecular mechanism for its elaboration and its role in the pathophysiology of ED.

CHAPTER 6

CHAPTER 6

88 kDa protein in other Ocular and Systemic Inflammatory Diseases

Introduction

Chapter 1 describes about identification, purification and characterization of novel 88 kDa protein from serum and vitreous of patients with ED. Since 88 kDa protein resembled in functions to that of APR, further studies were conducted to identify its expression in other ocular and systemic inflammatory diseases, which mimic ED such as uveitis, parsplanitis, ocular sarcoidosis, ocular toxoplasmosis and systemic diseases such as tuberculosis, leprosy, hepatitis and rheumatoid arthritis.

Results

Frequency of 88 kDa protein in other retinal vasculitis conditions and systemic inflammatory diseases are given in Table 2. 88 kDa protein was expressed in 54 % of cases with ED in AV stage and 23 % in active posterior uveitis. 88 kDa expression was found in 25 % cases of borderline leprosy, 33 % of cases with lepromatous leprosy, 40% of the cases with tuberculosis and 20 % of the cases with rheumatoid arthritis. 88 kDa protein was not detected in retinal vascular conditions mimicking ED, such as parsplanitis, ocular sarcoidosis, toxoplasmosis or diabetic retinopathy. Also 88 kDa protein was not detected in viral hepatitis. Expression of 88 kDa protein in diseases such as uveitis, tuberculosis, leprosy and rheumatoid arthritis are shown in 7.5 % native PAGE (Fig. 1)

Table 1: Clinical and demographic data of patients and control subjects

Sl. No.	Disease	No. of cases	Age (years)/sex
1.	Healthy control	40	28 ± 12 (28/M, 12/F)
2.	Eales' Disease	100	34 ± 9 (All M)
3.	Posterior uveitis	34	39 ± 15 (34/M, 20/F)
4.	Parsplanitis	18	42 ± 11 (10/M, 8/F)
5.	Ocular sarcoidosis	10	39 ± 15 (4/F, 1/M)
6.	Toxoplasmosis	10	34 ± 7 (3/M, 2/P)
7.	Leprosy Borderline leprosy Lepramatous leprosy	 4 6	39 ± 9 (6F, 4 M)
8.	Tuberculosis (pulmonary)	10	29 ± 9 (7M, 3F)
9.	Diabetic Retinopathy Non proliferative Proliferative	 7 14	59 ± 11(12M, 9F)
10.	Hepatitis (viral)	8	37 ± 9 (All M)
11.	Rheumatoid arthritis	5	33 ± 17 (3 M, 2F)

All values are mean ± SD. M: Male, F: Female.

Patients Selection

The clinical and demographic data of the patients are given in Table 1. ED was diagnosed after detailed fundus examination by an ophthalmologist. Differential diagnosis of ED was done as described earlier. Differential diagnosis of uveitis and parsplanitis was done as described by George et al (1996). All the uveitis cases included in this study did not have precise etiology and they were considered to be idiopathic. Serum samples and tissue sections from patients with tuberculosis were obtained from Tuberculosis Research Center, Chennai, India. Similarly specimens from leprosy patients were obtained from, Central Leprosy Research Institute, Chengalpattu, Tamil Nadu, India. Diagnosis of ocular sarcoidosis was made after doing biochemical investigation such as measurement of serum angiotensin converting enzyme (ACE). Final diagnosis of ocular scarcoidosis was made by correlating the laboratory tests with clinical features. Toxoplasmosis was diagnosed by correlating the clinical features with ELISA for detection of antibodies to *Toxoplasma* antigen. ELISA for HbsAg was done to diagnose the cases of viral hepatitis, which was supported by biochemical liver function tests. All the patients at the time of blood collection were not on any steroid or other medical treatment.

Table 2: Frequency of 88 kDa protein expression serum in various conditions

Sl. No.	Disease	No. of cases	Presence of 88 kDa protein	Frequency (%)
1.	Healthy control	40	0	0
2.	Eales' Disease	100	55	55
3.	Posterior uveitis	34	8	23
4.	Active Parsplanitis	18	0	0
5.	Ocular sarcoidosis	7	0	0
6.	Toxoplasmosis	5	0	0
7.	Leprosy			
	Borderline leprosy	4	1	25
	Lepromatous Leprosy	6	2	33.3
8.	Tuberculosis (pulmonary)	10	4	40
9.	Diabetic Retinopathy			
	Non proliferative	7	0	0
	Proliferative	14	0	0
10.	Rheumatoid arthritis	5	1	20
11.	Viral hepatitis	8	0	0

Figure 1

7.5 % native PAGE profile of serum samples. Lane 1; Eales' disease, lane 2; healthy volunteers, lane 3; parsplanitis, sarcoidosis or diabetic retinopathy, lane4; posterior uveitis, lane 5; tuberculosis, lane 6; leprosy, lane 7; rheu[illegible]; lane 8; toxoplasmosis, lane 9; viral hepatitis. Presence of [illegible] protein in serum samples of patients with Eales disease, tuberculosis, leprosy and rheumatoid ar[illegible] also indicated by arrow. The molecular weight markers for native PAGE was procured from Sigma Chemical Company (St Louis, MO, U.S.A). The arrow indicates the presence of 88 kDa protein in ocular and systemic inflammatory conditions. 88 kDa protein runs in β_1 glycoprotein region. α_2 MG; alpha 2 macroglobulins, β_2MG; beta 2 macroglobulins, β_1GP; beta 2 glycoprotein, HP; haptoglobin, β_1GP; beta 1 glycoprotein, TF; transferrin, CP; ceruloplasmin, LMGP; low molecular weight glycoproteins, PA; pre albumin.

Figure 2

Western blot analysis of 88 kDa Eales' protein in inflammatory conditions. Lane 1 serum sample from healthy volunteers. Lane 2 and 3; serum samples from patients with Eales disease, lane 4; serum sample from patient with tuberculosis, lane 5; serum sample from patient with lepromatous leprosy Lane 6; serum sample from patient with borderline leprosy, Lane 7; serum sample from patient rheumatoid arthritis and Lane 8; Purified 88 kDa protein from serum of patients with Eales' disease. Molecular weight markers are indicated at the left of the figure.

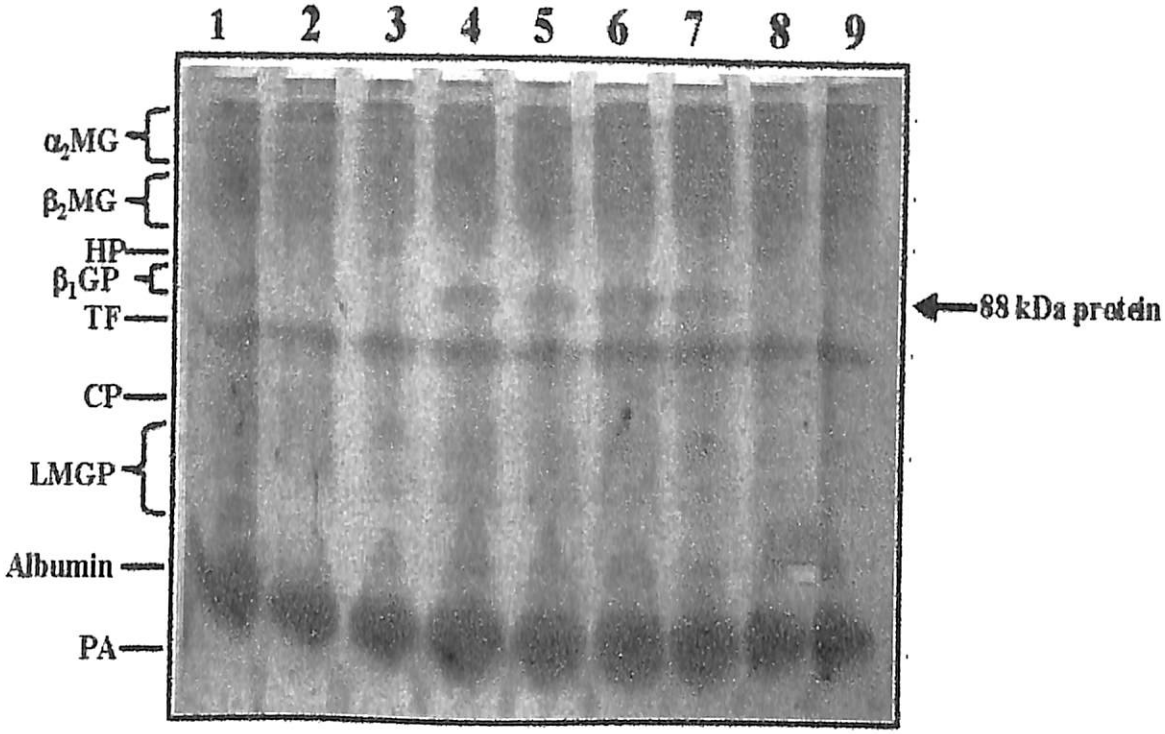


Figure. 1

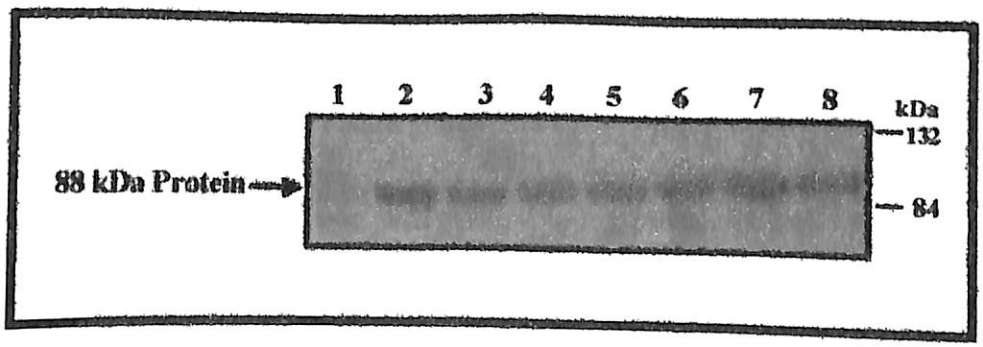


Figure. 2

Immunological identity of 88 kDa protein

When the 88 kDa protein in serum samples of patients with uveitis, tuberculosis, leprosy and rheumatoid arthritis, respective samples were subjected to Western blot analysis, single bands were observed, suggesting that 88 kDa protein found in ED and other diseases were immunologically identical. The results are shown in (Fig. 2)

Immunolocalization of 88 kDa in tissue sections obtained from patients with tuberculosis and leprosy

Four tissue sections from cutaneous granulomatous lesions from tuberculosis patients were subjected to immunohistochemical (IHC) analysis to find out whether 88 kDa protein is expressed in the local site of inflammation? Results showed that 88 kDa was expressed in 3 out of 4 cases of cutaneous tuberculosis patients (Fig. 3B). Expression was found in the giant cells. In these cases 88 kDa was also present in serum. However, immunoreactivity of 88 kDa protein was not detected in lesions from leprosy patients (Fig.3D).

Discussion

Since 88 kDa protein falls in the rank and file of acute phase reactant as it resembles in structure and function like other acute phase reactants , the expression of 88 kDa protein in other ocular and systemic inflammatory conditions were investigated. Results showed that 88 kDa protein was expressed in serum from patients with tuberculosis, leprosy and rheumatoid arthritis. The patients were referred to ophthalmologists for detailed fundus examination. Evaluation revealed that they did not have any signs or symptoms of intraocular inflammation and their vision was normal.

Figure 3

Immunolocalization of 88 kDa protein in tissue sections obtained from patients with tuberculosis and leprosy. (A) Hematoxylin and eosin staining of skin biopsy section obtained from a patient with tuberculosis revealed caseous necrosis and granulomatous change, with typical presence of giant cell (arrow). (B) Immunolocalization with anti 88 kDa antibody (goat polyclonal) revealed strong immunoreactivity in the cytoplasm of giant cells (arrow) in tissue section obtained from a patient with tuberculosis. (C) Hematoxylin and eosin staining of skin biopsy section obtained from a patient with leprosy, revealed caseous necrosis and granulomatous change, with typical presence of giant cell (arrow). (D) Immunolocalization with anti 88 kDa antibody (goat polyclonal) did not revealed significant immunoreactivity in the tissue section from a patient with leprosy. Magnification for A,B,C and D are X100, X200, X100 and X400 respectively.

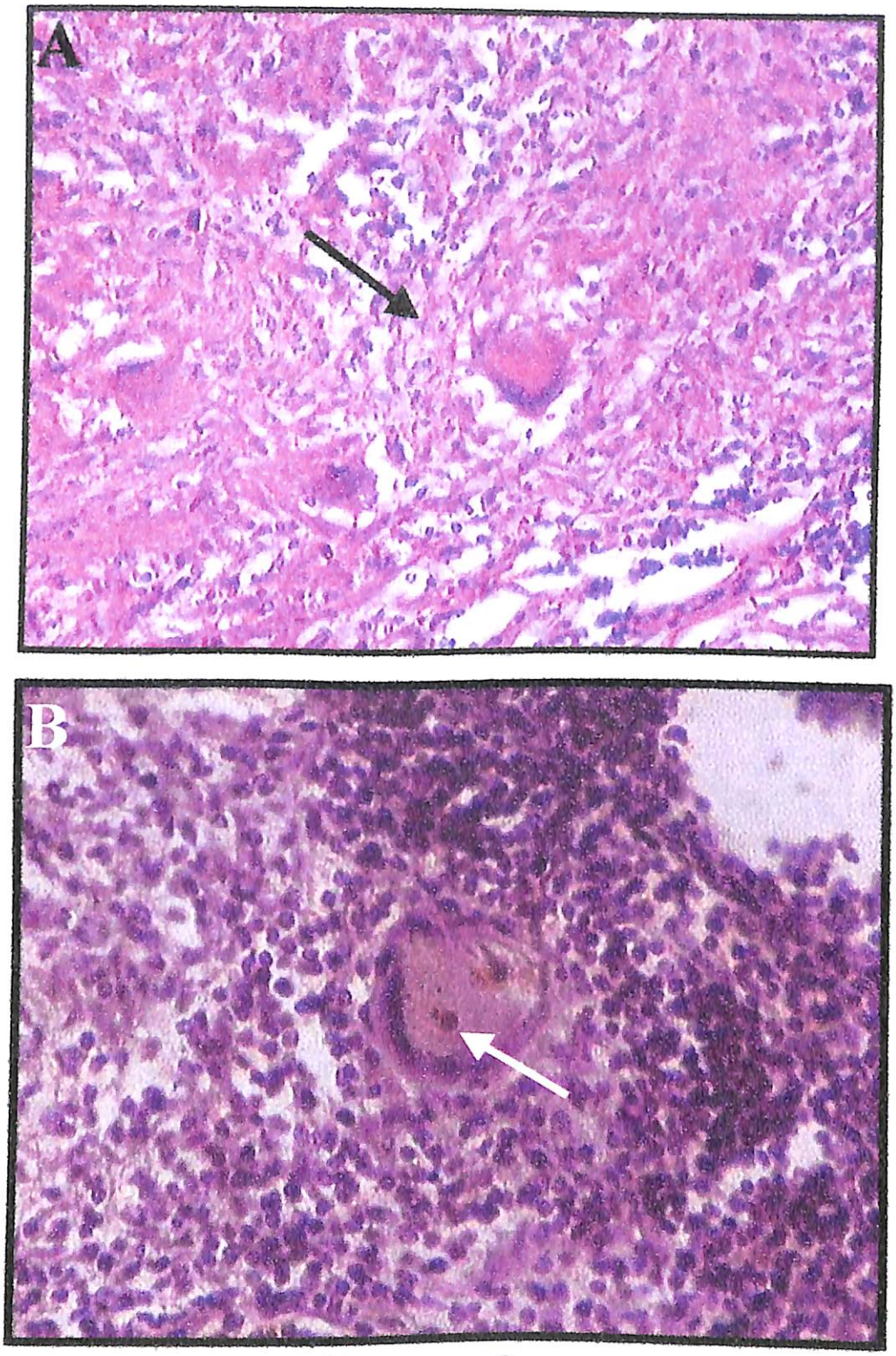


Figure. 3

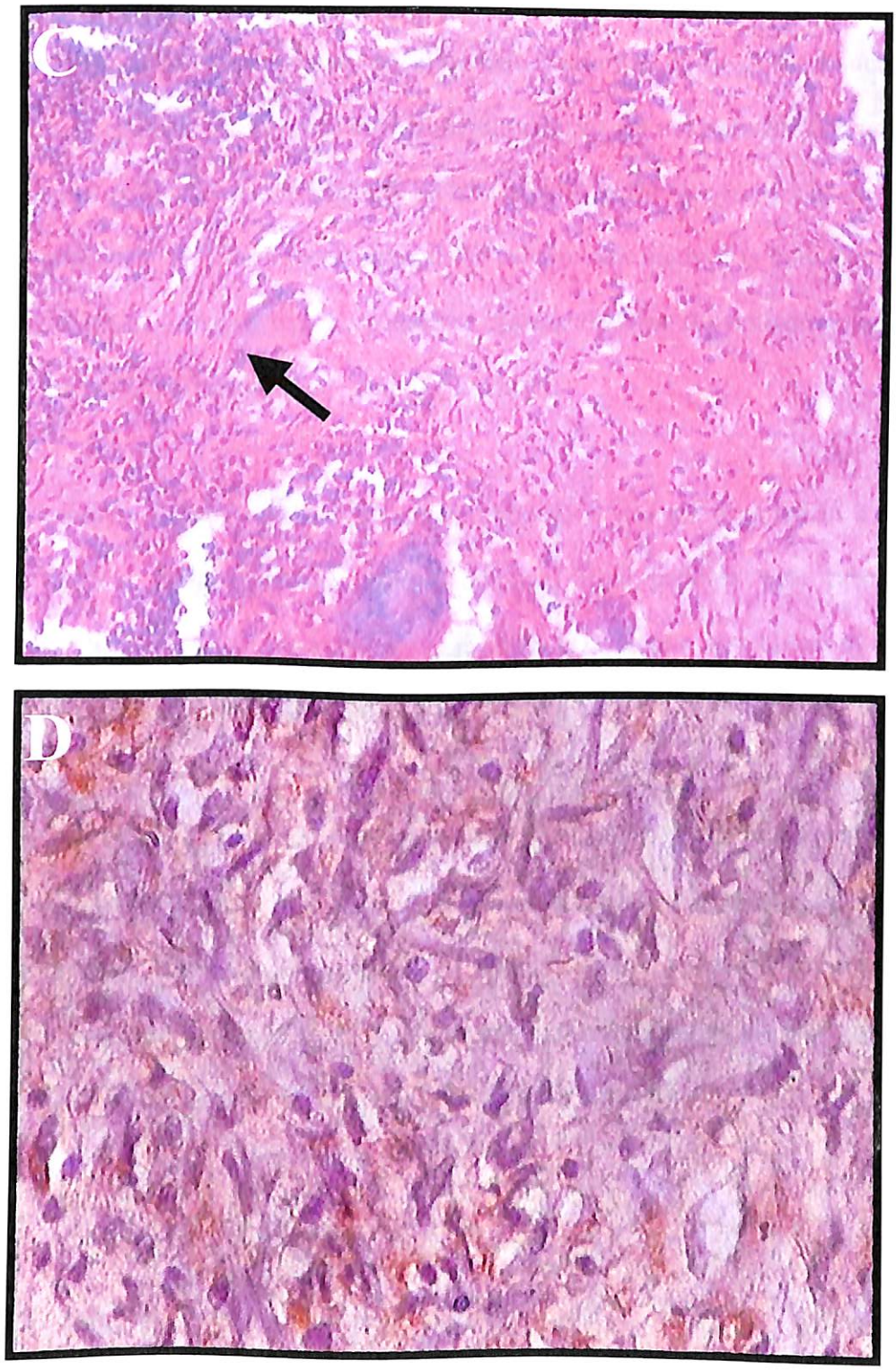


Figure 3

One does not have an answer at present as to why the 88kDa protein is expressed in patients with tuberculosis, leprosy and rheumatoid arthritis? Why all the patients with ED, uveitis, tuberculosis, leprosy or rheumatoid arthritis do not have 88-kDa protein in their serum? At first it was thought that 88 kDa protein might be specifically involved in the pathogenesis of ED. However from the results of this study it is believed that this protein might be a novel acute phase reactant expressed during inflammatory conditions, which has not been described earlier in the literature.

Other intriguing observation is that 88 kDa expression was not detected in 2 out of 4 tissue sections form cutaneous lesions obtained from patients with tuberculosis. Moreover in those cases, where expression of 88 kDa protein was observed in tissue sections, 88 kDa protein could were not detected in serum.

On the other hand immunoreactivity for 88 kDa protein was not detected even in single tissue section of patients with leprosy. The reason for such discrepancy and polymorphic variation in 88 kDa protein expression in different diseases remains as an enigma at present. However cloning of 88 kDa protein might shower light on the exact role of this protein in the pathomechanism of these inflammatory diseases.

SUMMARY
&
CONCLUSIONS

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- In the present study, for the first time Electron Spin Resonance Spectrometer (ESR) technique has been used to detect hydroxyl radical ($OH^{\bullet}$) in monocytes (MC) of patients with Eales' disease. Results showed that MC from patients with ED generated higher amounts of $OH^{\bullet}$, with diminished SOD activity and plasma GSH levels. In addition to the above, there was enhanced TBARS accumulation, with concomitant increase in iron content and diminished zinc content in MC of patients with ED, when compared with healthy control subjects. The production of $OH^{\bullet}$ was significantly inhibited, when Eales' disease MC were co-incubated with phorbol ester and iron chelators such as desferrioxamine and diethylenetriaminepentaacetic acid (DTPA). Similarly TBARS accumulation was markedly reduced when the MC from patients with Eales' disease co-incubated with phorbol ester and iron chelators. In vitro viability of Eales' disease MC was lower than healthy control MC, when each respective cells were incubated with their homologous serum. The viability of healthy MC was decreased, when incubated with Eales' disease serum, whereas healthy control serum prolonged the viability rate of Eales' disease MC. All these results revealed concomitant activation and recruitment of MC by $OH^{\bullet}$, with diminished antioxidant defense system. Finally all these events eventually result in enhanced free radical generation, which could amplify the inflammatory cascade leading to the retinal tissue damage in patients with Eales' disease.

- Phagocyte generated RNS formed through over expression of iNOS, have been implicated in mediating tissue damage associated with various inflammatory vasculopathies. In the present study, the possible involvement of phagocyte generated RNS in promoting RV and NV in ED was investigated. Results showed that there was significant increase in the expression of iNOS, 3NYTR accumulation, diminished SOD activity, elevated TBARS, iron and copper and decreased zinc content in MC of patients with Eales' disease, when compared with healthy control subjects. The elevated levels of ROS and RNS products correlated with diminished antioxidant status in patients with ED. In addition to the above, strong immunoreactivity for iNOS and 3NTYR was observed in inflammatory cells and in the endothelium of ERM obtained from patients with Eales' disease.

- Formation of carbonyl groups has been considered as early biomarker for oxidative protein damage in several human diseases. Plasma protein carbonyl content was elevated in patients with Eales' disease, when compared with control subjects. Increase presence of carbonyl group in Eales' disease correlated with diminished erythrocyte SOD activity and plasma GSH content. Increased TBARS accumulation also correlated with diminished SOD activity in erythrocytes of patients with Eales' disease.

- Determination of 8-OHdG has been considered as early marker to assess the severity of oxidative stress prevailing in the inflammatory conditions. In the present study, 8-OHdG has been determined in the leukocytes of patients with Eales' disease and in

healthy volunteers. Results showed that there was increase in the formation of 8-OHdG, in patients with Eales' disease, when compared with healthy control subjects. These results correlated with diminished SOD activity and GSH content.

- From the above results it is concluded that oxidative stress plays a pivotal role in promoting and amplification of retinal vasculitis and its sequlae, which could result in visual loss in patients with Eales' disease. Antioxidant supplementation has been shown to ameliorate oxidative stress induced tissue in several human diseases. Hence based on the above studies clinical trial is recommended to in future to evaluate the efficacy of antioxidant vitamins supplementation, iNOS inhibitors or iron chelators in controlling retinal vasculitis in patients with Eales' disease.

- A new 88 kDa protein has been identified in serum and vitreous of patients with Eales' disease. This protein was purified and its physiochemical properties were studied. N terminus and internal peptide sequences revealed no homology with the proteins described in the public databases. Hence 88 kDa protein was considered as novel protein. Biochemical properties resembled to some of the acute phase reactants (APR) like haptoglobin and transferrin (i.e has iron sequestering function, where by it acts as antioxidant). But there was no sequence homology of 88 kDa proteins with the above mentioned APRs. However, it is presumed that 88 kDa could be a novel APR elaborated in Eales' disease to protect the retina against oxidative damage. In order to further characterize the precise role of 88 kDa protein, it requires cloning , over

expression and complete cDNA sequence. Probably, this will help in developing diagnostic kit to detect the inflammatory conditions.

- Since 88 kDa protein was considered as APR, its expression was investigated in diseases which mimic Eales' disease. In addition to this, 88 kDa expression was investigated in other systemic inflammatory conditions such as rheumatoid arthritis, viral hepatitis and leprosy. 88 kDa expression was observed in serum of patients with uveitis, tuberculosis, leprosy and rheumatoid arthritis. 88 kDa protein found in these diseases were found to be immunologically identical with that of 88 kDa protein found in patients with Eales' disease. However, further studies are needed to delineate the role for the expression of 88 kDa protein in these inflammatory diseases.

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PUBLICATIONS
&
PAPER PRESENTATIONS

Manuscripts under consideration

1. **Rajesh M**, Sulochana KN, Coral K, Punitham R, Biswas J, Babu K, Ramakrishnan S. Determination of carbonyl groups content in plasma proteins as a useful marker for assessing the impairment in antioxidant defence in patients with Eales' disease. **Ind J Ophthalmol, 2003.**

2. **Rajesh M**, Ramesh A, Ravi PE, Balakrishnamurthy P, Coral K, Punitham R, Sulochana KN, Biswas J, Ramakrishnan S. Accumulation of 8 hydroxyguanosine and its relationship with antioxidant parameters in patients with Eales' disease: implications for antioxidant therapy. **Current Eye Research, 2003.**

3. **Rajesh M**, Sulochana KN, Ramakrishnan S, Biswas J, Manoharan PT. Involvement of mononuclear macrophage in the inflammation and oxidative stress in patients with Eales' disease. **Experimental Eye Research, 2003.**

Paper Presentations

1. **M. Rajesh, K.N. Sulochana, S. Ramakrishanan and P.T. Manoharan** Further Evidence that Oxidative Stress may be a Risk Factor Responsible for the Development of Retinal Vasculitis in Eales' disease. **SERI -ARVO, Singapore** (Abstract:109 pp.119) 7th February 2003.

2. **M. Rajesh, K.N. Sulochana and S. Ramakrishnan** Involvement of Peripheral Blood Mononuclear Cells in the Oxidative Stress and Retinal Inflammation in patients with Eales Disease – 71st **SOCIETY OF BIOLOGICAL CHEMISTS (INDIA)** Annual meeting, Ludhiana, Punjab from 14th - 16th November 2002 (Abstract: P2A-2, pp.88).

3. **M. Rajesh, K.N. Sulochana and S. Ramakrishnan** Increased expression of iNOS, diminished SOD activity and Zn, accumulation of 3-Nitrotyrosine, TBARS, Fe and Cu in peripheral blood mononuclear cells of patients with Eales disease: Further studies on the disease mechanism- **ARVO, USA**, May 2002, (Abstract:B501)

4. **M. Rajesh, K.N. Sulochana and S. Ramakrishnan** Molecular evidence for involvement of reactive nitrogen species in the pathogenesis of retinal vasculitis in Eales' disease - Apogee-2002, 20th all India academic work in Bits Pilani from 12th to 14th March 2002.

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Purification and Characterization of a Novel 88 kDa Protein from Serum and Vitreous of Patients with Eales' Disease

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Eales' disease is a perivasculitis that affects the peripheral retina of young adults and results in recurrent vitreous hemorrhage. Although increased oxidative stress and decreased antioxidant defense have been reported to be associated with Eales' disease, the exact cause for the disease and its pathogenesis are not known. Here is reported the identification, purification and characterization of a new protein from the serum and vitreous of patients with Eales' disease. This protein was purified using preparative electrophoresis and HPLC. The purified protein had a retention time of 9·2 min in RP HPLC. Its molecular weight as determined by gel permeation chromatography was 88 kDa hence, it was termed as 88 kDa protein. Alcian blue and Schiffs staining revealed 88 kDa protein to be a glycoprotein. Proteins purified from both serum and vitreous exhibited anti lipid peroxidation effect on erythrocyte when added during in vitro assay of thiobarbuteric acid reactive substances (TBARS). In addition to this property the protein also has Fe^{2+} sequestering effect. The anti TBARS activity of 88 kDa protein was completely inhibited by 0·1 mM concentration of parachlromercuric benzoate (PCMB) and 5,5′ dithiobis(2-nitrobenzoic acid) DTNB. The total thiol content (cysteine) of the purified 88 kDa protein was found to be 8% by mass. Eighty eight kDa protein from both the sources namely vitreous and serum are immunologically identical when studied using polyclonal antibodies raised in goat against purified serum protein. The N terminal sequence of 88 kDa protein by automated Edman's degradation chemistry is A D D P N S L S P S A F A E A L A L L R D S X L A R F V. The protein and DNA data base search revealed no match to 88 kDa protein and hence this was considered as unique protein. Further knowledge on the in vivo function of 88 kDa protein is very important to understand its role in the pathogenesis of Eales' disease.

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Key words: Eales' disease; retinal perivasculitis; intraocular inflammation; 88 kDa protein; antioxidant.

1. Introduction

Eales' disease is an idiopathic inflammatory venous occlusion that primarily affects the peripheral retina of young adults (Eales, 1880, 1882). It is rare in developed countries like the U.S.A., but common in the Indian subcontinent with an incidence of one in 200–250 ophthalmic patients (Puttana, 1970). The disease predominantly affects males in the age group of 20–40 years and is often bilateral. The peak age of onset of symptoms is 20–30 years. The clinical symptoms in most patients include intraocular inflammation, recurrent vitreous hemorrhage, and retinal neovascularization. Bleeding from neovascularization is common, usually recurrent and is one of the major causes for visual loss in such cases (Das et al., 1994). Vitreous hemorrhage is a prominent manifestation of this disease and it is the major cause of visual impairment in patients suffering from Eales' disease. In addition, the formation of epiretinal membrane, which imposes a tractional pull on the retina causing retinal detachment. Timely intervention with pars-plana vitrectomy and laser photocoagulation and removal of epiretinal membrane helps in the partial restoration of vision. The treatment for Eales' disease is based on the symptoms. It includes corticosteroids, photocoagulation with or without retinal cryoablation and vitrectomy at various stages of the disease process (Das et al., 1994).

Despite rigorous research at various centers in the country, the etiology of the disease remains unknown. Among the several diseases or systemic abnormalities found to be associated with Eales' disease, tuberculosis has been favoured by many, however none has shown any evidence of direct infection of the retina or eye by tuberculosis bacteria. Although, a recent report from our centre demonstrated the presence of *Mycobacterium tuberculosis* DNA by nested PCR technique in surgically removed epiretinal membranes from patients with Eales' disease (Madhavan et al., 2000). Several biochemical studies have been done on the serum and vitreous samples of patients with Eales' disease. The important findings includes the

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demonstration of increased levels of α globulin and presence of a 23 kDa protein in serum (Pratap, Metira and Gupta, 1976; Rengarajan, Muthukarrupan and Namperumalasamy, 1989).

The biochemical studies carried out earlier by our group had revealed increased oxidative stress, and decreased antioxidant status in the blood and vitreous samples of the individuals suffering from Eales' disease (Bhooma et al., 1997; Sulochana, Biswas and Ramakrishnan, 1999). In this article the presence of a new protein designated as 88 kDa in serum and vitreous of patients with Eales' disease is demonstrated. Details of methods used for the purification of the 88 kDa protein and its physiochemical properties are also presented.

2. Materials and Methods

Chemicals

All the fine chemicals used in this study were from Sigma Chemical Company (St. Louis, MO, U.S.A.) unless specified and all other reagents were of the highest analytical grade.

Subjects and Samples Used for the Study

Eales' disease was diagnosed after detailed fundus examination by an ophthalmologist. The case was then included on the basis of the following criteria: primary periphlebitis of the retina, neovascularization and vitreous hemorrhage not associated with anterior uveitis, choroiditis, parsplanitis, diabetes mellitus, cataract, glaucoma, optic atrophy, corneal opacities, macular degeneration or sarcoidosis.

Other causes for retinal perivasculitis such as syphilis, tuberculosis were eliminated by performing respective routine immunological and biochemical investigations. Ten subjects with diabetes mellitus and ten non-diabetic (normal) individuals contributed their serum samples for the protein characterization studies. Samples of vitreous from diabetic subjects, removed during vitrectomy as part of their treatment were also used in the study. The vitreous aspirate from donor eyeballs, after removing the cornea, was also subjected to analysis.

For purification of this protein, undiluted vitreous and serum samples were used. Blood samples were collected from confirmed cases of Eales' disease when they came to the clinical lab for routine investigation and for getting physician's clearance before vitrectomy surgery. The vitreous aspirate obtained during surgery was transported to the biochemistry research laboratory for immediate processing of the sample.

All human studies were performed following the guidelines of the Helsinki declaration and were approved by the Institution's Review Board. Informed consent was obtained from all patients and volunteers before including them in the study.

Protein Purification

The serum and vitreous samples were subjected to protein electrophoresis, to assess the protein profile. Samples showing the presence of 88 kDa protein were stored at −20°C until they were subjected to purification. Storage period varied between 15 days to 1 month. The new protein was identified by running the samples on 7·5% native polyacrylamide gel electrophoresis (Davis, 1964) After electrophoresis the gel was subjected to silver stain (Morrissey, 1981). In a similar experiment the gel was stained with periodic acid—Schiff's reagent and Alcian blue (Wardi and Michos, 1972; Gradilone, Arranz and Cabada, 1997) respectively for identifying the glycoprotein.

Serum samples from six to eight patients were pooled (1·5 gm protein per 25 ml serum) and subjected to 70% ammonium sulfate precipitation. The precipitate was dialysed against 10 mM phosphate buffer pH 7·4, for 48 hr with frequent changes of buffer at every 4–6 hr interval. After dialysis, the sample was centrifuged at 5000 rpm for 30 min at 4°C, the supernatant was subjected to further purification by preparative gel electrophoresis. At this stage, protein estimation was done by Lowry's method (Lowry et al., 1951). Preparative electrophoresis (BioRad 491, Hercules, CA, U.S.A.) was done on a 5% PAGE and the proteins were eluted using 0·05 mM Tris–glycine buffer, pH 8·3. Fractions of 5 ml were collected at a flow rate of 1 ml min^{-1} using continuous buffer elution system (BioRad Echno PumpEPI). All the fractions were screened at 280 nm on a spectrophotometer (Beckman DU 640, Fullerton, CA, U.S.A.) for protein content. They were also subjected to slab gel electrophoresis and stained by silver stain. The fractions positive for 88 kDa protein were further subjected to anti TBARS activity. The active fractions were pooled and stored at −20°C until further purification. During every stage of purification, the presence of the protein was confirmed by performing 7·5% native PAGE along with positive serum obtained from patients with Eales' disease.

HPLC Purification

Active fractions from preparative electrophoresis were pooled and protein content was measured and then concentrated by lyophilization using Virtis freeze drier (NY, U.S.A.) The protein concentrate (50 mg) was further purified using ODS reversed phase column in LKB HPLC (details of column dimensions were given in the legend to Fig. 2). Briefly the column was equilibrated using 10 mM phosphate buffer with 0·1 M sodium chloride pH 7·4 Then after injection the protein was eluted isocratically with the aforementioned buffer containing 50% methanol at the flow rate of 0·2 ml min^{-1}. The elution was monitored at 280 nm using LKB Bromma UV detector (model no:

2151, Sweden). The eluted fractions were subjected to anti TBARS activity and active fractions were pooled and rechromatographed in the same conditions until the protein resolved as a homogenous peak. The purity was further checked by SDS–PAGE electrophoresis followed by silver staining. The purified protein was dialysed against 10 mM HEPES buffer pH 7·4, and used for further characterization studies.

Purification of 88 kDa protein from vitreous was done by adopting the same procedure used for serum, but for the omission of ammonium sulfate precipitation. The undiluted vitreous samples (4·0 ml) from six patients were pooled, homogenized with phosphate buffered saline pH 7·4 and centrifuged at 5000 rpm for 30 min at 4°C. The protein content in the supernatant was measured by the method of Lowry et al. (1951). The supernatant (3 ml) was directly subjected to 5% preparative polyacrylamide gel electrophoresis. The fractions were analysed as per serum. The active fractions were pooled and purified further by HPLC. The purified protein was dialysed against 10 mM HEPES buffer pH 7·4 and then used for characterization. All these procedures were done in a cold room maintained at 4°C.

Anti TBARS Activity

The anti TBARS activity was measured using the method of Ledwozywa, Stepien and Kadziolka (1986). The extent of lipid peroxidation in fresh erythrocytes was measured as TBARS. The results were expressed as nmol of malondialdehyde (MDA)dL^{-1} RBC. The quantity of the product, MDA was decreased when the assay was performed in the presence of 100 ng of the purified 88 kDa protein to 1 ml of erythrolysate. This property was used to identify the presence of 88 kDa protein during its purification in fractions collected from preparative electrophoresis and HPLC. The same assay was carried out to assess the effect of various thiol inhibitors such as PCMB and DTNB.

Molecular Weight Determination

This was carried out using a gel permeation HPLC column (TSK 3000, Amersham Pharmacia) and Sigma molecular weight markers, along with these markers, 2 μg of purified protein was loaded to gel filtration column. The proteins were eluted with 10 mM phosphate buffer pH 7·4 at a flow rate of 0·2ml min^{-1}. The chromatography was monitored at 280 nm (LKB Bromma UV detector). Molecular weight was calculated from the graph plotted with retention time against log molecular weight [Fig. 3(A)].

Amino Acid Sequencing and Protein Database Analysis

The purified protein was subjected to 12% SDS–PAGE electrophoresis and electrotransferred to polyvinylidine difluoride membrane (PVDF, Millipore Co., Bedford, MA, U.S.A.). The transferred protein was visualized by staining the membrane with 0·1% ponceau S (dissolved in 1% acetic acid in water). The protein band was then carefully excised, destained with several changes of milli Q water. Then the PVDF membrane containing the protein was directly subjected to N terminal sequencing by using automated Edman degradation chemistry (Matsudaria, 1987) in Applied Biosystems 470A gas phase amino acid sequencer (Foster City, CA, U.S.A.). The derived sequence was analysed by protein and DNA data base analysis using SWISS Prot, and NCBI programs.

Biochemical Characterization

Eighty eight kDa protein was associated with Eales' disease where ocular inflammation exists. Eighty eight kDa protein was presumed to be a stress/acute phase protein and thus tested for any functional property for transferrin, ceruloplasmin and haptoglobin. Transferrin activity in the purified protein was done by measuring iron (Carter, 1971) and iron binding capacity (ICSH, 1978). The results were expressed as μg of iron dL^{-1} serum. Ceruloplasmin activity was examined by its oxidation of *p*-phenylendiamine (Ravin, 1961) while haptoglobin activity was assessed by its agglutination property on RBCs (Herbert, 1978).

While performing the titration of H_2O_2 with $KMnO_4$, addition of 88 kDa protein reduced the level of H_2O_2, hence glutathione peroxidase activity was suspected and tested by the method of Rotruck et al. (1973). As 88 kDa protein has anti TBARS activity, it was thought to have antioxidant property. The protein was thus tested for superoxide dismutase (SOD) activity by following the method of Misra and Firdovich (1972). The total thiol content of the purified 88 kDa from serum and vitreous was determined by using Ellman's (1959) reagent 5,5′ dithiobis (2-nitrobenzoic acid) (DTNB).

Immunological Studies on 88 kDa Protein

Partial purification of IgG from anti serum. One mg of purified 88 kDa protein was used for raising polyclonal antibody in goat as described earlier (Chase, 1967). The antibody was custom made by Chemicon Inc. (Temecula, CA, U.S.A.). Purification of IgG from polyclonal antibody was done by adopting the method described by Talwar (1967). This essentially involved salt fractionation using sodium sulphate (18%) followed by ion exchange chromatography on DEAE column. The immunoglobulins were eluted using 100 mM phosphate buffer pH 8·0. This fraction was used for further analysis.

Immunoelectrophoresis. Immunoelectrophoresis was performed using the method described by Culliford (1964) and Lowrell (1972). Briefly, 1% agarose was dissolved in 0·04 M Veronal buffer pH 8·6. The same

buffer was used as tank buffer. Electrophoresis was carried out for 2 hr with 10 mA per slide. Following electrophoresis, another horizontal trough was made in between the two antigen wells and filled with 0·1 ml of partially purified antibody (1 : 500). The slides were then kept in a humid chamber at room temperature for 24–36 hr to allow the passive diffusion of antigen and antibody. Precipitin arcs were viewed directly under dark background, the slides were then dried completely (60°C for 2 hr) and subsequently stained with Coomassie brilliant blue (R250) for permanent documentation.

Western blot. The purified 88 kDa protein (20 μg) from serum and vitreous was subjected to 12 % SDS–PAGE. Then the proteins were electrotransferred to nitrocellulose membrane (Millipore Co., Bedford, MA, U.S.A.). The membrane was blocked with 5 % skimmed milk in PBS pH 7·4 for 1 hr at room temperature in a rocking platform. The membrane was then probed with anti 88 kDa antibody (goat polyclonal 1 : 500 dilution). Mouse anti goat IgG antibody was used as secondary antibody (Sigma, St. Louis, MO, U.S.A. 1 : 1000 dilution) and the protein was visualized using NBT/BCIP as a substrate (Brunette, 1981).

3. Results

Physiochemical Properties of 88 kDa Protein

A new protein has been identified in serum and vitreous of patients with Eales' disease. In 7·5 % native gel electrophoresis, it resolved in the α_2 globulin region. Samples of serum from patients with type II diabetes and normal controls did not show the presence of this protein (Fig. 1). Alcian blue and periodic acid Schiffs stain independently revealed this protein to be a glycoprotein (data not shown).

This protein has been purified to homogeneity as revealed by HPLC (Fig. 2). In reversed phase HPLC this new protein had a retention time of 9·2 min. Steps involved in the purification and the yield of protein from serum and vitreous are presented in Table I. The apparent molecular weight of the protein

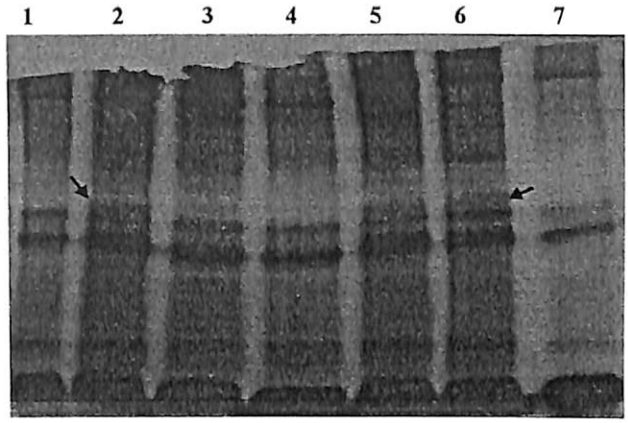

FIG. 1. Native PAGE (7·5 %) stained by Commassie brilliant blue G250. Lanes 1 and 7, serum from normal healthy volunteer, lanes 2, 5 and 6 are serum from patients with Eales' disease. Lanes 3 and 4 serum from patients with diabetic retinopathy. The presence of 88 kDa protein in lanes 2, 5 and 6 is indicated by arrow in the photomicrograph. Twenty μg of serum protein was uniformly used for protein profile.

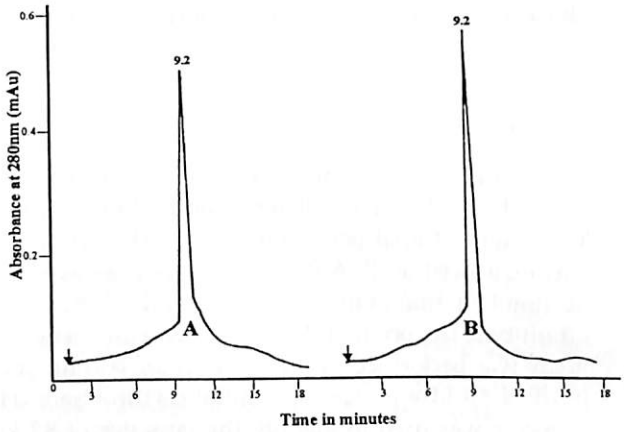

FIG. 2. HPLC elution profile showing the homogeneity of the 88 kDa protein from (A) serum and (B) vitreous (5 μg each) with retention time of 9·2 min. The separation was performed using RP C18 column 5 μm particle size with 300 Å pore size (Lichrosphere, Merck, Germany).

as determined by gel filtration HPLC was 88 kDa [Fig. 3(A)]. Hence it is designated as 88 kDa Eales' protein. Native PAGE (7·5 %) of the purified protein

TABLE I

Purification of Eales' protein from serum and vitreous

Purification step	Serum		Vitreous	
	Protein (mg)	Yield %	Protein (μg)	Yield %
Undiluted pooled sample	1500 (15 ml)	100	2000 (4 ml)	100
60 % $(NH_4)_2$ SO_4 precipitation	700	46	–	–
5 % preparative gel electrophoresis	50	3·3	100	5
RPHPLC	1·5[a]	0·1	20[a]	1

Protein estimation up to step 3 was made by Lowry method, while HPLC with BSA as standard was used in the last step.
[a]Single peak in HPLC (Fig. 2).

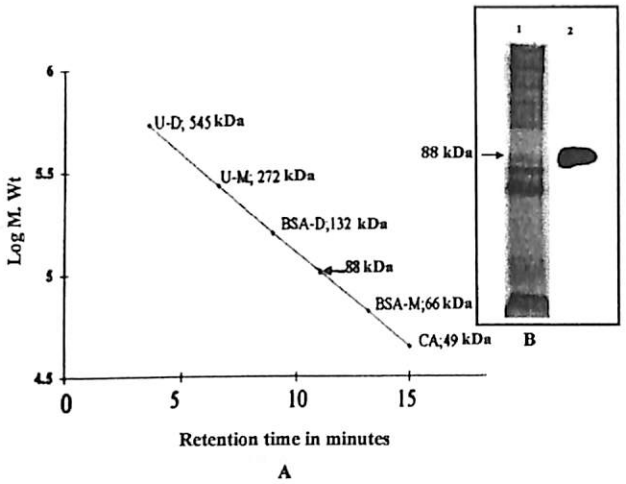

FIG. 3. (A) Molecular weight determination of the purified protein. This was done using gel filtration column (TSK 3000, Amersham Pharmacia, Sweden) using LKB Bromma HPLC system. The gel permeation molecular weight markers used were urease dimer (545 kDa), urease monomer (272 kDa), BSA dimer (132 kDa), BSA monomer (66 kDa) and carbonic anhydrase (49 kDa). (B) Native PAGE (7·5 %) of purified protein stained by silver nitrate indicating its homogeneity. Lane 1, serum (20 μg) from patient with Eales' disease, lane 2, purified 88 kDa protein (30 μg).

followed by silver stain revealed the purity of this protein [Fig. 3(B)].

Anti TBARS activity of 88 kDa Protein

Eighty eight kDa protein had anti TBARS activity in the in vitro assays. This property was used routinely for its identification besides PAGE electrophoresis during purification steps. The anti-TBARS activity was dose dependent on protein concentration. It was tested for this effect on erythrocytes of patients with diabetes and without diabetes and also patients with Eales' disease (Table II). Both serum and vitreous derived 88 kDa protein showed similar extent of anti TBARS activity. The conventional thiol group inhibitors namely DTNB and PCMB inhibited this activity of the protein, indicating the importance of thiol residues in its function (Table V). This was further confirmed by the fact that cysteine residues contributed 8 % of total mass of the protein. The in vitro demonstration of anti TBARS activity in neutral pH is suggestive of the fact that the contribution by carbohydrate moiety is negligible as enolization of sugar (in alkaline pH) is needed for antioxidant function.

Iron Binding Capacity

The purified 88 kDa (either 1 or 2 μg) when incubated with 1·0 ml of human serum before estimating free iron (Fe^{+2}) and iron binding capacity (IBC) found to decrease the level of iron and IBC. This effect was dose dependent on the concentrations of 88 kDa protein used. The results are summarized in Table III.

Amino Acid Sequencing

The N-terminal sequence of 88 kDa protein is given in Table IV. The sequence homology search on database revealed no match to this protein. Hence 88 kDa protein was considered as a novel protein. In physiochemical properties it resembled certain other body proteins. The common properties and the differences between 88 kDa protein and other similar proteins are given as a comparative chart in Table VI.

Immunoelectrophoresis and Western Blot

Results of these experiments are illustrated in Figs 4 and 5). Western analysis showed that the purified 88 kDa protein from serum and vitreous were homogeneous. Immunoelectrophoresis revealed that 88 kDa protein from serum and vitreous were immunologically identical. In addition to this Western analysis of serum and vitreous samples from patients with diabetic retinopathy or normal healthy volunteer (only serum) revealed the absence of 88 kDa protein indicating the specificity of the polyclonal antibody produced against 88 kDa protein (data not shown).

TABLE II

Effect of purified 88 kDa protein on erythrocytes TBARS activity

Condition	TBARS levels of RBC alone (nm MDA dL^{-1})*	TBARS levels of RBC in the presence of 88 kDa protein (mmMDAdL^{-1})*		% decrease in erythrocytes TBARS activity in the presence of 88 kDa protein	
		+ 100 ng of protein	+200 ng of protein	100 ng of protein	200 ng of protein
Eales' ($n = 10$)[a]	2930 ± 282	2154 ± 154	1874 ± 131	25·6	48
Diabetes ($n = 10$)[b]	566 ± 28	449 ± 19	372 ± 19	20·7	34
Normal control ($n = 10$)[c]	405 ± 33	344 ± 25	286 ± 21	15	30

*The values are in mean ± S.E..
[a]Age: 20–40 years in Eales' group (all males).
[b]Age: 40–60 years in diabetes (eight men, two females).
[c]Age: 15–45 years in control group (six men, four females).

TABLE III

Effect of 88 kDa protein on serum iron and total iron binding capacity

Serum constituents (μgm dL^{-1})	No. 88 kDa protein	Purified 88 kDa protein	
		1 μg per 1 ml serum	2 μg per 1 ml serum
Fe^{+2}	193 ± 22	100 ± 16	82 ± 13
Total iron binding capacity (as Fe^{+2})	484 ± 36	286 ± 28	214 ± 20

The values are expressed as mean ± S.E. of five individual experiments. All the samples were from normal volunteers (two males and three females) of age group 25–40 years.

TABLE IV

Sequence of 28 amino acids from N-terminus of 88 kDa protein

1																							
Ala	-	Asp	-	Asp	-	Pro	-	Asn	-	Ser	-	Leu	-	Ser	-	Pro	-	Ser	-	Ala	-	Phe	-
A		D		D		P		N		S		L		S		P		S		A		F	
Ala	-	Glu	-	Ala	-	Leu	-	Ala	-	Leu	-	Leu	-	Arg	-								
A		E		A		L		A		L		L		R									
																							28
Asp	-	Ser	-	X	-	Leu	-	Ala	-	Arg	-	Phe	-	Val									
D		S		X		L		A		R		F		V									

Concentional one letter symbols are used for amino acids.
X—not determined.

TABLE V

Effect of thiol inhibitors on the anti TBARS activity of 88 kDa protein

Inhibitors	Concentration (mM)	Anti TBARS activity Relative activity (%)
None	–	100%
DTNB	0·05	25
	0·1	Nil
PCMB	0·05	37
	0·1	8

The results were average of three independent experiments.

4. Discussion

It is known that during inflammation certain acute phase proteins will be elaborated and are seen in circulation. Some of these proteins include haptoglobin, transferrin, hemopexin and ceruloplasmin. Acute phase proteins generally play a defensive role. For example, haptoglobin in combination with hemoglobin has peroxidase effect and scavenges hydrogen peroxide. Transferrin binds with ferrous ion in order to arrest Fenton's reaction while, ceruloplasmin has ferroxidase property i.e. conversion of ferrous ion to ferric ion. It is recently reported that neural retina and photoreceptor cells were found to synthesize mRNA

TABLE VI

Comparative table of 88 kDa protein with other similar proteins

Protein	Common property	Different from 88 kDa Eales' protein
Transferrin	Binding with iron Acute phase protein	Molecular weight (77 kDa) Not a glycoprotein No homology in N terminus
Haptoglobin	Iron binding, Hemoglobin binding, Agglutination, Electrophoretic movement (α_2 region) raised in inflammation, mol. wt 90 kDa	Seen in all individuals irrespective of their disease. No sequence homology with 88 kDa protein in N terminus
Ceruloplasmin	Iron binding, acute phase protein feroxidase activity, endogenous antioxidant	Not having ceruloplasmin activity with paraphenylene diamine. No sequence homology in N terminus
Superoxide dismutase	Endogenous antioxidant	But did not inhibit epinephrine auto oxidation (No SOD activity)
Glutathione peroxidase (GP_x)	Removes H_2O_2	88 kDa does not require GSH for activity, while GP_x needs GSH for its activity

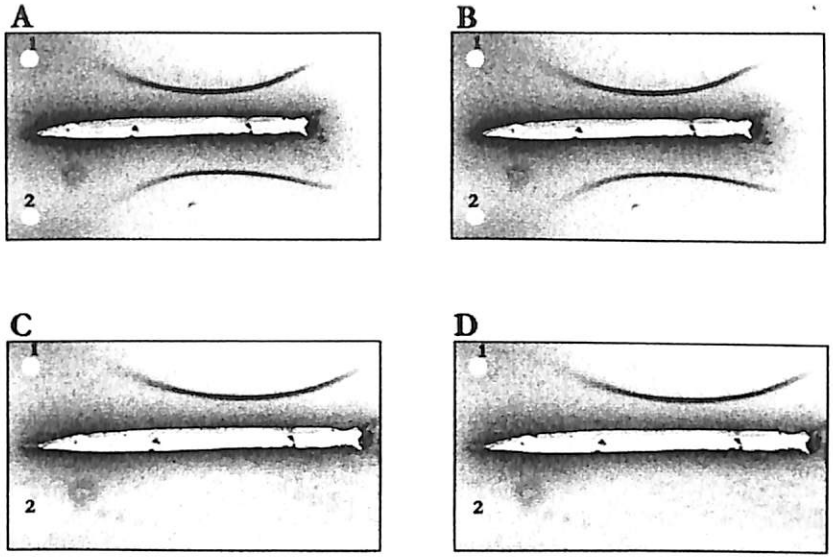

FIG. 4. Immunoelectrophoresis of serum 88 kDa protein with Eales' disease. (A) Well 1 serum (60 μg) from patient with Eales' disease, well 2 vitreous (10 μg) from patient with Eales' disease. (B) Purified 88 kDa protein from vitreous (2 μg, well 1) and serum (2 μg, well 2) from patients with Eales' disease. (C) Well 1 vitreous (60 μg of protein) from a patient with Eales' disease, well 2 vitreous (10 μg of protein) from patient with diabetic retinopathy. (D) Well I serum (60 μg of protein) from patient with Eales' disease, well 2 serum (10 μg of protein) obtained from normal healthy volunteer. In all the above experiments (A–D), the central well was probed with anti 88 kDa antibody (goat polyclonal 1 : 500 dilution.

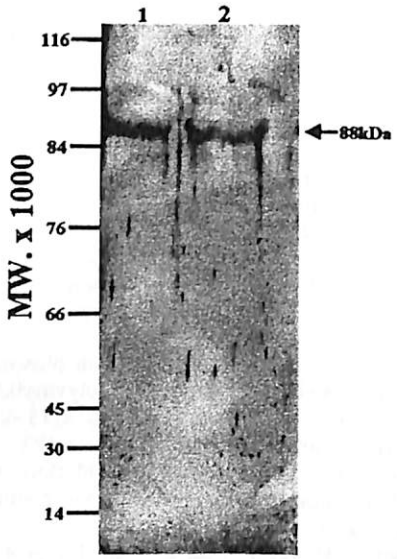

FIG. 5. Western blot for purified 88kDa protein. Lane 1, 30 μg purified 88 kDa from serum. Lane 2, 30 μg purified 88 kDa from vitreous.

for both haptoglobin and hemopexin molecules (Weiheng et al., 1998). Moreover haptoglobin has been found to be expressed locally in retina and it is different from that of haptoglobin found in serum. This is valid since serum haptoglobin cannot enter the retina, unless there is a breakdown of blood retinal barrier (Weiheng et al., 1998). In this study, increased levels were found of the newly found 88 kDa protein in vitreous when compared with serum. These observations support, the view that the 88 kDa protein may also be of ocular origin and have a defensive role. It is interesting to know that 88 kDa protein from serum and vitreous were found to be immunologically identical when tested using antibodies raised against 88 kDa protein (purified from serum) in goat.

Glycoxidation and lipoxidation are two detrimental pathways recently recognized to have an important role in causing tissue damage (Wolf, Zyjiang and Hunt, 1991; Baynes and Thorpe, 1999). Certain oxidative markers such as carboxylmethyllysine [CML, an advanced glycation end product (AGE)] have been found in diseases associated with oxidative stress (Baynes and Thorpe, 1999). Such molecules have been found to accumulate in epiretinal membranes surgically removed from patients with Eales' disease (Sulochana, Nagaraj and Mruthinti, 2000). Also found were elevated levels of CML in serum samples obtained from patients with Eales' disease (Coral et al., 2000). Both glycoxidation and lipoxidation are mediated through Fe^{+2}. In this context the magnitude of the prevention of accumulation of AGE products may be directly proportional to the extent of Fe^{+2} sequestering effect by 88 kDa protein. This was evident from the iron binding assays done in this study. Simultaneous quantification of 88 kDa protein and TBARS levels in patients with Eales' disease in different stages of disease process may be useful to know its in vitro function.

Wu and Rao (1996) have reported the presence of a retinal protective protein in autoimmune uveitis and showed it to be as similar to transferrin. In another report the same authors have recommended antioxidant therapy in the form of a free radical scavenger like vitamin B, C and iron chelators in uveitis to overcome the oxidant stress to retina (Rao, Wu and Geetha, 1995).

The fact that elevated levels of 88 kDa protein in vitreous than in serum have been observed, indicates that 88 kDa protein in circulation might have reached the retina due to the breakdown of the blood retinal barrier during inflammation or this protein might have been produced locally. Moreover, vitreous is considered to be the molecular repository for retinal metabolism (Reddy, 1979). However immunohistochemical studies in BRM are underway in the laboratory to find out the exact cellular site of its production in retina. In addition to this elevated TBARS have also been observed in patients with Eales' disease and 88 kDa protein is also elevated in this condition. Careful animal experiment may help in understanding the exact in vivo kinetics of its production, turn over and molecular role in the pathogenesis of Eales' disease. Another interesting aspect in a majority of patients with Eales' disease, the damage is caused only in the peripheral retina and not in the central retina (Das et al., 1994). The reason for the same is not clear at present. Future studies on 88 kDa protein could throw light on the mechanism of the pathogenesis in Eales' disease.

In conclusion, a novel protein from patients with Eales' disease has been identified, purified and partially characterized. It appears from our results presented in this article that 88 kDa protein may be a protective molecule secreted in order to defend the retina against oxidative stress.

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The authors thank Dr Erdjan Salih, Department of Orthopedic Surgery, Children's hospital, Boston, MA, U.S.A. for his kind help in amino acid sequencing of the 88 kDa protein and Dr Narasingh A. Rao, Doheney Eye Institute, School of Medicine, University of Southern California, Los Angeles, CA, U.S.A. for his generous help in preparing antisera to 88 kDa protein.

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Presence of a 88 kDa Eales protein in uveitis, tuberculosis, leprosy and rheumatoid arthritis

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
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Summary

Background: Eales disease (ED) is an idiopathic retinal vasculitis affecting young adult males. We have earlier reported the identification, purification and partial characterization of a novel 88 kDa protein found in the serum of patients with ED. The aim of the present study was to look for the 88 kDa protein in serum samples obtained from cases of retinal vasculitis mimicking ED and in other systemic inflammatory diseases.

Material/Methods: Serum samples from healthy volunteers and from patients with ED, uveitis, parsplanitis ocular sarcoidosis, toxoplasmosis, leprosy, diabetic retinopathy, viral hepatitis, and rheumatoid arthritis were analyzed for the presence of the 88 kDa protein by polyacralymide gel electrophoresis (PAGE). The immunological identity of the 88 kDa protein found in ED and in other diseases was investigated by Western blot. Immunohistochemistry was performed on epiretinal membranes (ERM) obtained from ED patients to localize the 88 kDa protein.

Results: 88 kDa protein were detected in serum samples obtained from patients with posterior uveitis, tuberculosis, leprosy and rheumatoid arthritis. The 88 kDa protein found in serum from patients with ED is immunologically identical to that found in other systemic inflammatory conditions. 88 kDa protein was localized in inflammatory cells and in nonvascular endothelium in ERMs obtained from patients with ED.

Conclusions: We have identified a novel acute phase reactant, which is elaborated in ocular and systemic inflammatory conditions other than Eales disease. Further work is necessary to decipher the precise role of the 88 kDa protein in the pathophysiology of these inflammatory diseases.

key words: 88 kDa protein • tuberculosis • leprosy • rheumatoid arthritis

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BACKGROUND

Eales disease (ED) is an idiopathic retinal perivasculitis, which affects young adult males in the age group of 20–40 years [1]. Clinical features include inflammation (peripheral retinal vasculitis), ischemic changes (retinal capillary non-perfusion), and neovascularization in the retina or at the disk, which often leads to vitreous hemorrhage with or without retinal detachment [2]. Though ED was first described in Western countries, for unknown reasons the disease is now more common in the Indian subcontinent [3].

We have earlier reported the identification, purification and characterization of a novel 88 kDa protein found in serum and vitreous samples collected from patients with ED [4]. There are several retinal vascular diseases which mimic the clinical features seen in ED [5]. In addition to these findings, there have been several reports associating tuberculosis with ED [6-8]. Recently Madhavan et al. have detected the presence of *Mycobacterium tuberculosis* gene by polymerase chain reaction (PCR) in epiretinal membranes (ERMs) obtained from patients with ED [9]. There are also reports that ED may be associated with systemic diseases such as leprosy, rheumatoid arthritis and viral hepatitis [5]. However, there are no reports on the successful isolation of viable *Mycobacterium tuberculosis* in culture from specimens obtained from patients with ED. Hence we were interested in knowing whether the 88kDa protein was elaborated in serum from patients with tuberculosis, leprosy, rheumatoid arthritis and viral hepatitis.

MATERIAL AND METHODS

All the fine chemicals used in this study were procured from Sigma Chemical Company (St Louis, Missouri, USA) unless otherwise specified.

Table 1. Clinical and demographic data of patients and control subjects.

Sl. No.	Disease	No. of cases	Age (years)/sex
1	Healthy controls	40	28±12 (28/M, 12/F)
2	Eales disease	100	34±9 (All M)
3	Posterior uveitis	34	39±15 (19/M, 15/F)
4	Parsplanitis	18	42±11 (10/M, 8/F)
5	Ocular sarcoidosis	10	39±15 (4/F, 1/M)
6	Toxoplasmosis	10	34±7 (3/M, 2/F)
7	Leprosy		
	Borderline leprosy	4	39±9 (6F, 4M)
	Lepramatous leprosy	6	
8	Tuberculosis (pulmonary)	10	29±9 (7M, 3F)
9	Diabetic retinopathy		
	Non-proliferative	7	59±11 (12M, 9F)
	Proliferative	14	
10	Hepatitis (viral)	8	37±9 (All M)
11	Rheumatoid arthritis	5	33±17 (3M, 2F)

All values are mean±SD;
M –Male; F –Female

We recruited all new ED cases from January 2000 to February 2001. Protocols involving human subjects strictly adhered to the tenets of the Helsinki Declaration. The first author's Institutional Research Cell and Ethical Committee approved this study.

The clinical and demographic data of the patients are given in Table 1. ED was diagnosed after detailed fundus examination by an ophthalmologist. Differential diagnosis of ED was performed as described elsewhere [5]. The differential diagnosis of uveitis and parsplanitis was performed as described by George et al [10]. None of the uveitis cases included in this study had a precise etiology, and may be considered idiopathic.

Serum samples from new cases of pulmonary tuberculosis were kindly provided by Dr M. S. Jawahar, (Senior Physician, Tuberculosis Research Center, Chennai, India). Dr D. Sekar (Pathologist and Microbiologist, Central Leprosy Research Institute, Chengalpattu, Tamil Nadu, India) provided serum samples from patients with borderline and lepramatous leprosy. Diagnosis of ocular sarcoidosis was made after biochemical investigations, such as measurement of serum angiotensin converting enzyme (ACE). ACE levels were more than 40 units in the cases included in this study (normal up to 30 units [11]). The final diagnosis of ocular scarcoidosis was made by correlating the laboratory tests with clinical features. Toxoplasmosis was diagnosed by correlating the clinical features with ELISA for detection of antibodies to *Toxoplasma* antigen. ELISA for HbsAg was done to diagnose the cases of viral hepatitis, which was supported by biochemical liver function tests. All the patients at the time of blood collection were not taking steroids or other medications. The healthy volunteers chosen in this study were neither smokers nor habitual alcohol drinkers, and they did not have any systemic diseases revealed by medical examination.

The serum was prepared from clotted blood. Serum samples were stored in aliquots at –70°C, and were kept frozen until use and thawed only once. Prior to freezing, the protein content in the serum samples was determined by the Lowry method using BSA as standard [12].

The serum was screened for the 88 kDa protein by loading 25 µg serum protein onto 7.5% native PAGE. Electrophoresis was carried out at 150V for an hour, using running buffer (0.025 M Tris and 0.92 M glycine, pH 8.3). After electrophoresis was complete, the gels were stained with 0.025% coomassie brilliant blue R250 and then destained with 40% methanol and 7% acetic acid. The protein profiles in the gels were documented using a gel documentation system (Image Master, VDS, Amersham Biosciences, UK).

For Western blot analysis, 75 µg of serum was subjected to 7.5% native PAGE. Resolved proteins on the gels were then electrotransfered to nitrocellulose membrane (Millipore Co, Bedford, Massachusetts, USA). The membrane was blocked with 5% skimmed milk in phosphate buffered saline (PBS), pH 7.4, for 1 hr at room

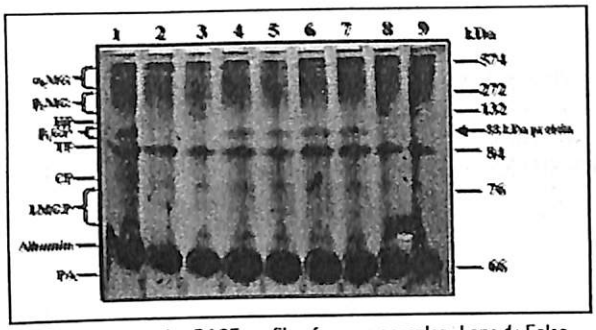

Figure 1. 7.5% native PAGE profile of serum samples. Lane 1; Eales disease, lane 2; healthy volunteers, lane 3; parsplanitis, sarcoidosis or diabetic retinopathy, lane 4; posterior uveitis, lane 5; tuberculosis, lane 6; leprosy, lane 7; rheumatoid arthritis; lane 8; toxoplasmosis, lane 9; viral hepatitis. Presence of 88 kDa protein in serum samples of patients with Eales disease, tuberculosis, leprosy and rheumatoid arthritis is indicated by arrow. The molecular weight markers for native PAGE were procured from Sigma Chemical Company (St Louis, Missouri, USA). The arrow indicates the presence of 88 kDa protein in ocular and systemic inflammatory conditions. 88 kDa protein runs in β_1 glycoprotein region. α_2 MG – alpha 2 macroglobulins, β_2 MG – β_2 macroglobulins, β_2 GP – beta 2 glycoprotein, HP – haptoglobin, β_1 GP – beta 1 glycoprotein, TF – transferrin, CP – ceruloplasmin, LMGP – low molecular weight glycoproteins, PA – pre albumin.

temperature. Subsequently the membranes were probed with anti 88 kDa antibody (goat polyclonal, affinity purified, 1:500 dilution, specificity of the antibody characterized as elsewhere described [4]) and incubated at room temperature for two hours. After this step, the membranes were again washed in PBS pH 7.4 and then incubated with anti goat IgG antibody conjugated with alkaline phosphatase used as secondary antibody (Santa Cruz Biotechnology, California, USA, 1:1000) for an hour and the protein was visualized using nitroblue tetrazolium/5-bromo 4-chloro indoyl phosphate (NBT/BCIP) as the substrate [13].

Surgically excised ERMs from six patients with ED were used for immunohistochemical analysis to find out the precise cellular location of the 88 kDa protein. The tissues were processed and embedded in paraffin wax blocks. 5 µm sections were cut using a microtome (Leica, JUNG RM 2045, Germany) and the paraffin sections were transferred on to poly-L-Lysine coated glass slides. After deparafinization, the sections were incubated with 3% H_2O_2 for 10 min to quench the endogenous peroxidase activity. The sections were washed in PBS, pH 7.4, and exposed to 2% normal goat serum solution (Dako, Copenhagen, Denmark) for 30 min at 4°C. The sections were then washed in PBS and probed with primary antibody, anti 88 kDa (goat polyclonal, 1:200) for 2 hrs at room temperature in a humid chamber. After this step, the sections were washed in PBS and then incubated with biotinylated rabbit anti goat IgG antibody (Dako) for 45 min, followed by incubation in avidin-biotin complex (Dako) for 15 min at room temperature. The final color was developed with 3,3'diaminobenzidine (DAB) substrate kit (Dako). For histologic correlation, the sections were counterstained in hematoxylin for 45 seconds, after which the slides were mounted (Entellan, Merk, Darmstadt, Germany) and coverslipped. Photographic documentation (ASA 100 film, Eastman Kodak, Rochester, New York, USA) was performed with an optical microscope (Nikon, Japan) at 200X and 400X magnifications [14].

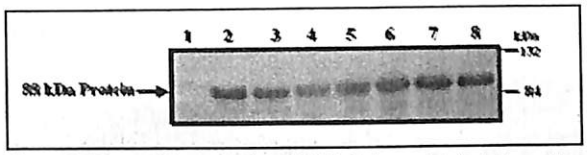

Figure 2. Western blot analysis of 88 kDa Eales protein in inflammatory conditions. Lane 1: serum sample from healthy volunteers. Lanes 2 and 3: serum samples from patients with Eales disease, lane 4: serum sample from patient with tuberculosis, lane 5: serum sample from patient with lepromatous leprosy, lane 6: serum sample from patient with borderline leprosy, lane 7: serum sample from patient rheumatoid arthritis, and lane 8: purified 88 kDa protein from serum of patients with Eales disease. Molecular weight markers are indicated at the left of the figure.

Table 2. Frequency of 88 kDa protein expression serum in various conditions.

Sl. No.	Disease	No. of cases	Presence of 88 kDa protein	Frequency (%)
1	Healthy controls	40	Nil	0
2	Eales disease	100	55	55
3	Posterior uveitis	34	8	23
4	Active parsplanitis	18	Nil	0
5	Ocular sarcoidosis	7	Nil	0
6	Toxoplasmosis	5	Nil	0
7	Leprosy			
	Borderline leprosy	4	1	25
	Lepromatous leprosy	6	2	33.3
8	Tuberculosis (pulmonary)	10	4	40
9	Diabetic retinopathy			
	Non-proliferative	7	Nil	0
	Proliferative	14	Nil	0
10	Rheumatoid arthritis	5	1	20
11	Viral hepatitis	8	Nil	0

RESULTS

The frequency of occurrence of the 88 kDa protein in retinal vasculitis conditions other than ED and in systemic inflammatory diseases are given in Table 2. The 88 kDa protein was expressed in 55% of cases with ED and 23% of patients with posterior uveitis. We were able to detect 88 kDa expression in 25% of the cases of borderline leprosy, 33% of the cases with lepromatous leprosy, 40% of the cases with tuberculosis, and 20% of the cases with rheumatoid arthritis. The 88 kDa protein was not detected in retinal vascular conditions mimicking ED, such as parsplanitis, ocular sarcoidosis, toxoplasmosis, or diabetic retinopathy. Also, the 88 kDa protein was

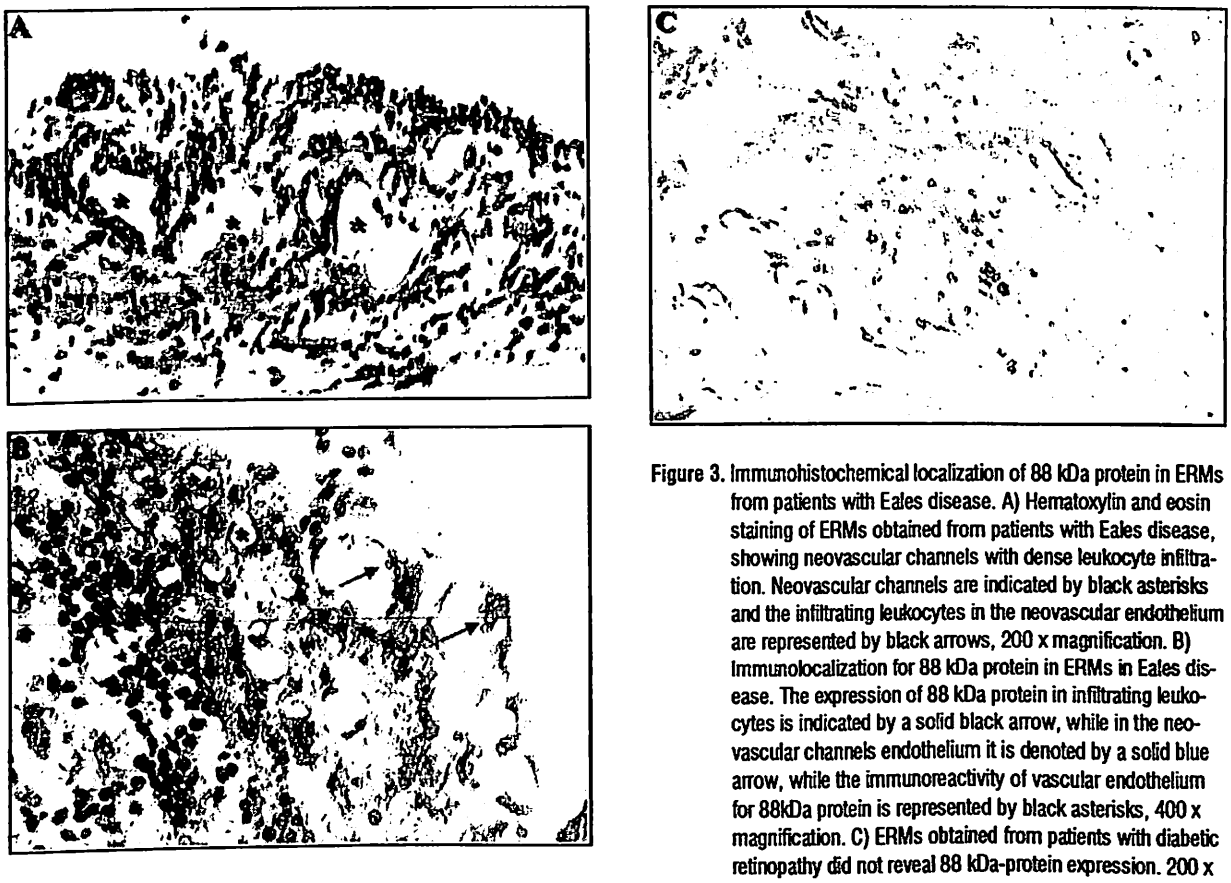

Figure 3. Immunohistochemical localization of 88 kDa protein in ERMs from patients with Eales disease. A) Hematoxylin and eosin staining of ERMs obtained from patients with Eales disease, showing neovascular channels with dense leukocyte infiltration. Neovascular channels are indicated by black asterisks and the infiltrating leukocytes in the neovascular endothelium are represented by black arrows, 200 x magnification. B) Immunolocalization for 88 kDa protein in ERMs in Eales disease. The expression of 88 kDa protein in infiltrating leukocytes is indicated by a solid black arrow, while in the neovascular channels endothelium it is denoted by a solid blue arrow, while the immunoreactivity of vascular endothelium for 88kDa protein is represented by black asterisks, 400 x magnification. C) ERMs obtained from patients with diabetic retinopathy did not reveal 88 kDa-protein expression. 200 x magnification.

not detected in viral hepatitis. The expression of the 88 kDa protein in diseases such as uveitis, tuberculosis, leprosy and rheumatoid arthritis are shown in 7.5% native PAGE (Figure 1)

When we detected 88 kDa protein in serum samples of patients with uveitis, tuberculosis, leprosy, and rheumatoid arthritis, respective samples were subjected to Western blot analysis, where purified 88 kDa protein from serum was used as positive control. Single bands in all the lanes were observed, suggesting that the 88 kDa proteins found in ED and other diseases were immunologically identical. The results are shown in Figure 2.

Figure 3A illustrates the Hematoxylin and Eosin staining of ERMs from ED patients, showing neovascular channels with infiltration of leukocytes. Immunoreactivity for anti 88 kDa antibody was detected in infiltrating leukocytes, neovascular channels, and endothelium (Figure 3 B) in all the ERMs studied (n = 6). On the other hand, ERMs from patients with diabetic retinopathy (n = 8) did not reveal the presence 88 kDa protein expression (Figure 3 C).

DISCUSSION

Our earlier study revealed the presence of 88 kDa protein in vitreous samples from patients with ED [4]. It was shown that, during intra ocular inflammatory conditions, the blood retinal barrier breaks down, followed by seepage of circulating blood into the retina, which eventually results in amplification of the inflammatory reaction [15].

Immunolocalization studies for the 88 kDa protein in ERMs obtained from ED patients revealed that the 88 kDa protein is localized in the infiltrating leukocytes, capillary endothelium, and the neovascular channels. This could be due to the breakdown of the blood retinal barrier, which resulted in the invasion of leukocytes into the ERM. Therefore, the 88 kDa protein seen in ERMs in ED may have originated from the circulating blood.

We also found 88 kDa protein in serum from patients with tuberculosis, leprosy, and rheumatoid arthritis. The patients were referred to ophthalmologists for detailed fundus examination. Evaluation revealed that they did not have any signs or symptoms of intraocular inflammation and their vision was normal. We do not have an answer at present as to why the 88kDa protein is expressed in patients with tuberculosis, leprosy and rheumatoid arthritis? Or why all the patients with ED, uveitis, tuberculosis, leprosy or rheumatoid arthritis do not have 88 kDa protein in their serum? Our initial theory was that 88 kDa protein might be specifically involved in the pathogenesis of ED. However, the results of this study have convinced us that this protein may be a novel acute phase reactant expressed during inflammatory conditions, not previously described.

However, a longitudinal study performed by following up patients who had the 88kDa protein prior to and after treatment could help us determine the possible role of this protein in the inflammatory diseases described in this manuscript. Efforts are also being made in our laboratory to clone this protein, so that we can further characterize its *in vivo* role and to delineate its involvement in the pathophysiology of those diseases where the expression of 88 kDa protein has been detected.

CONCLUSION

We have reported the appearance of a novel 88 kDa acute phase protein in serum of patients with uveitis, tuberculosis, leprosy and rheumatoid arthritis. Future work should be directed towards understanding the role of the 88 kDa protein in the pathogenesis of these diseases.

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CLINICAL BIOCHEMISTRY

Involvement of oxidative and nitrosative stress in promoting retinal vasculitis in patients with Eales' disease.

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Abstract

Objectives: Eales' disease (ED) is an idiopathic retinal vasculitis condition, which affects retina of young adult males. The histopathological hallmark in ED is the adhesion of leukocytes to the endothelium and the infiltration of these cells into the retinal parenchyma. Phagocyte generated free radicals have been implicated in mediating tissue damage associated with various inflammatory vasculopathies. In the present study, we have investigated the possible role of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in causing retinal tissue damage in ED.

Design and methods: 35 patients with ED and 20 healthy control subjects were included in the study. Monocytes (MC) were separated from peripheral blood of the respective study participants. Inducible nitric oxide synthase (iNOS) protein expression was assessed using Western blot and 3 nitrotyrosine (3NTYR) by reversed phase high performance liquid chromatography (RP HPLC). Thiobarbituric acid reactive substances (TBARS) were determined by measuring malondialdehyde (MDA) formed. Superoxide dismutase (SOD) activity was assayed based on the ability of SOD to inhibit autooxidation of epinephrine. Iron, copper and zinc content were determined using Atomic Absorption Spectrometer. Immunolocalization of iNOS and 3NTYR was performed on the surgically excised epiretinal membranes (ERM) from patients with ED.

Results: There was a significant increase in the expression of iNOS, as well as 3NTYR accumulation, diminished SOD activity, elevated lipid peroxides, iron, copper and decreased zinc content in the MC of patients with ED when compared with healthy control subjects. The elevated levels of ROS and RNS products correlated with diminished antioxidant status in patients with ED. Strong immunoreactivity for iNOS and 3NTYR was observed in inflammatory cells and endothelial cells in ERM obtained from patients with ED.

Conclusions: Our findings from this study clearly reveal the involvement of RNS and ROS in the development of retinal vasculitis in ED. Based on our present study and earlier studies we confirm the role of free radicals in mediating retinal tissue damage in ED. Hence we believe selective inhibition of iNOS or supplementation with antioxidants vitamin E and C might be beneficial in controlling retinal vasculitis in patients with ED. © 2003 The Canadian Society of Clinical Chemists. All rights reserved.

Keywords: Eales' disease; Retinal vasculitis; Monocytes; Inducible nitric oxide synthase; Nitrotyrosine; Superoxide dismutase; Lipid peroxides

1. Introduction

Eales' disease (ED) is an idiopathic inflammatory venous occlusion condition, which primarily affects the peripheral retina of young adult males. Clinical features in ED include perivascular phlebitis, nonperfusion and neovascularization. Loss of vision is due to recurrent vitreous hemorrhage and its sequlae [1]. ED has been reported from United States, United Kingdom and Canada in the later half of the 19th and early 20th century. But for unknown reasons, it is now rare in developed countries and more commonly seen in the Indian sub continent [2]. ED affects young adults in the age group of 15 to 45 yr with male predominance (99%) [2]. Our recent review provides comprehensive information pertaining to clinopathological features, clinical management and recent progress in the understanding of ED [3].

Inflammation is the main clinical feature in ED [4]. Inflammation involves predominantly the peripheral veins [4]. Intact internal limiting membrane (blood retinal barrier) is known to provide a barrier to the infiltration of circulating phagocytes in to the retina [5]. However, in response to

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inflammatory stimulus, there is disruption of blood retinal barrier, which results in increased vascular permeability associated with migration and invasion of circulating inflammatory cells into the retina [5]. Several histopathological studies in ED have revealed the infiltration of chronic inflammatory cells, especially lymphocytes and monocytes in the epiretinal membrane (ERM) around the neovascular channels [6–9].

Phagocyte generated ROS and RNS have been implicated in the tissue damage during various systemic diseases [10,11]. There is considerable evidence that reactive oxygen species (ROS) and RNS, particularly peroxynitrite ($ONOO^-$) can act as important mediators of inflammation, shock and ischemia/reperfusion injury [12]. In diseases where the role of ROS/RNS has been associated in its pathogenesis, selective inhibition of iNOS activity or supplementation with antioxidants, which quench these radicals, have been shown to ameliorate the inflammation induced tissue injury [13]. To our knowledge, there has been no human clinical research done on the role of ROS/RNS in development of retinal vasculitis (RV) in ED to date. Hence, our aim of the present study was to investigate whether the accumulation of RNS is involved in the development of retinal vasculitis (RV) damage in ED. We have chosen to work on peripheral blood mononuclear macrophage (PBMM), which is otherwise known as monocytes (MC) as the available clinical material, because it has been earlier shown that MC infiltration observed in ERM share the same cluster differentiating antigen (CD) marker with PBMM [8,9]. In addition to the above, MC harbors higher amounts of iNOS, and have been shown to play a major role in tissue remodeling during inflammation by its ability to produce and modulate the ROS/RNS activities [14].

2. Materials and methods

2.1. Chemicals

All fine chemicals were obtained from Sigma Chemical Co., St Louis, CA, U.S.A, unless specified. All other reagents were of the highest purity, and obtained from E-Merck, Mumbai, India. Water used in this study was purified using Millipore water purification system (Millipore Co., Bedford, MA, U.S.A).

2.2. Patients and controls

Thirty-five patients with ED (31 ± 11 yr, all males) were diagnosed after detailed fundus examination by an ophthalmologist. The patients' and control subjects' characteristics are given in (Table 1). The diagnostic tests performed for differential diagnosis of ED are listed in Table 2. RV in ED is usually characterized by the presence of serous exudate around the retinal veins with ill-defined margin, retinal edema and vitreous hemorrhage. Patients diagnosed with ED did not have any obvious systemic diseases, such as diabetes mellitus or any other systemic inflammatory disorders, which was revealed by their normal range for hematologic, biochemical and immunologic investigations. They were nonobese, nonsmoking and not habituated to alcohol consumption. Before blood collection, they were not on any medical treatment, including supplementation of antioxidant vitamins. After phlebotomy, they were treated with oral prednisone, 1 mg/kg body weight every day for one week and slowly tapered to 10 per week for 6 to 8 weeks. Twenty healthy adult volunteers (29 ± 10 yr, all males) with normal fundus from the Authors' Institute, were recruited for the study to have them as control. None of them were smokers or alcoholics or taking vitamin supplements.

Table 1
Characteristics of patients and control subjects

	Healthy control subjects	Eales' Disease	P value
n	20	35	–
Age (Years)	31 ± 11	29 ± 10	NS
Sex	All Males	All Males	–
Plasma post prandial glucose (mg%)[¶]	92 ± 2.3	96 ± 3.3	NS
Serum Alkaline phosphatase (u/L)[¶]	80 ± 4	81 ± 6	NS
Blood urea (mg %)[¶]	18 ± 0.6	17.5 ± 0.5	NS
Total protein (g %)[¶]	6.9 ± 0.8	7.1 ± 0.8	NS

Values are expressed as mean ± SD. Student's t test was employed to analyze the statistical significance of the data. $P < 0.05$ was considered statistically significant. [¶] These biochemical parameters were analyzed by semi-automated analyzer (Micro Lab 2000, E-Merck, India) using the commercially available kits (Bayer Diagnostics, Ltd., India).

Table 2
Diagnostic tests performed for diagnosis of Eales' disease

1	Routine Tests
	Complete Haemogram
	Erythrocyte sedimentation rate
	Blood glucose
	Blood chemistry
	Chest Radiograph
	Urine analysis
	C reactive protein
	Sickle cell disease screening test
2	Infectious Diseases
	VDRL
	TPHA
	Mantoux test
	Brucella agglutination test
3	Autoimmune disorders
	Antinuclear antibody
	Anti-double stranded DNA antibodies
4	Miscellaneous Tests
	Serum lysozyme assay
	Serum angiotensin converting enzyme

VDRL, venereal disease research laboratory; TPHA, *Treponema pallidum* hemeagglutination test.

The authors' Institutional Research Board and Ethics Committee approved this study. Written consent was obtained from all the participants. Experiments pertaining to human subjects were performed according to the tenets of Helsinki declarations. All the assays in ED and control samples were run in parallel. To ensure that findings were consistent and independent of experimental variations, we determined intra assay variation for all the parameters. The values are mentioned in the text wherever necessary.

2.3. Separation of monocytes

A phlebotomist aseptically collected 10 mL of venous heparinized blood sample from the study subjects and immediately transported to laboratory for further processing. The blood was diluted with equal volumes of PBS pH 7.4 and carefully overlaid on equal volume of ficoll-hypaque density gradient solution (Amersham Biosciences, UK) and centrifuged at 3000 rpm for 30 min at ambient temperature. After centrifugation, the interface which contains mononuclear cells (lymphocytes and monocytes) were carefully aspirated and washed three times with PBS pH 7.4 supplemented with antibiotic solutions (Hi Media, Mumbai, India). After the final step of washing, the pellets were carefully mixed with RPMI 1640 medium and were added to 35-mm-diameter tissue culture dish. The cells were allowed to adhere for 2 hrs at 37°C in a 5% CO_2 environment [15]. The adherent cell population contained at least 90 to 95% monocytes by microscopic examination after staining with Wright-Giemsa stain. Other cells appeared to be lymphocytes. More than 95% of the cells were viable as determined by trypan blue exclusion test [16]. MC separated in this manner was immediately processed for the investigations. 2×10^6 cells/mL were used for each investigation unless specified.

2.4. Western blot analysis of iNOS expression

Cells were suspended in PBS pH 7.4 containing protease inhibitors, sodium orthovanadate, phenyl methyl sulfonyl fluoride (PMSF) and aprotinin (each 10 μg/mL). The cells were then lysed using a sonicator (Vitris Inc., NY, USA) 15K for 15 s × 2 on ice. Then the lysates were clarified to remove cellular debris by centrifuging at 4,000 rpm for 10 min at 4°C. The protein content in the supernatant was determined by Lowry method [17] using BSA as the standard. 50 μg of protein was loaded on to a 9% SDS PAGE gel (Bio Rad, Richmond, CA, U.S.A). After electrophoresis, the proteins were transferred onto PVDF membranes (Amersham Biosciences, UK) using Bio Rad Trans blot equipment. The membrane was then blocked with 5% skimmed milk powder in PBS pH 7.4 for an hour at room temperature. After thorough washing of the membrane with PBS, it was subsequently incubated with iNOS antibody (1:1000, rabbit polyclonal, Santa Cruz biotechnology, CA, USA) over night at 4°C in rocking platform. Following this step, the membrane was again washed with PBS and probed with secondary antibody anti rabbit IgG HRP conjugate (1:2500, mouse monoclonal, Sigma Chemical Co., MO, U.S.A) for 2 hr at room temperature (RT). Then iNOS protein was visualized using diamino benzidine as previously described [18]. The amount of iNOS protein was quantified using an image documentation system (Image Master, Visual Documentation System, Amersham Biosciences, UK) and the values are expressed as pixel intensity/50 μg of protein.

2.5. Determination of 3NTYR

For quantification of 3NTYR by RP HPLC, Millipore water with 18 MΩ conductance (Millipore Co., Bedford, MA, U.S.A) was used. The water was further treated with 10 μM diethylenetriaminepentaacetic acid (DTPA) to remove iron, which is known to produce artifacts during protein hydrolysis and interfere in subsequent steps. In addition to this, the water treated in this way was further filtered through 0.22 μm filter (Millipore Co., Bedford, MA, USA), autoclaved and used for the experiments. 3NTYR were determined by following the method described by Crow and Ischiropoulos [19] with slight modifications. In brief, the proteins from cell lysates were resuspended in 6N HCl and hydrolyzed by heating at 110°C for 16hrs. Hydrolysates were ultrafiltered with 10 kDa cut off filters (Millipore Co., Bedford, MA, U.S.A). The acid was removed by lyophylization. The lyophylized powder was dissolved in 1.0 mL of treated sterile milli Q water and stored at −80°C until further analysis. For determination of tyrosine, 10 μL aliquot of the sample from the above preparation was derivatized (precolumn) with orthopthaldehyde (OPA) according to the manufacturers' instructions (Agilent Technologies, U.S.A). 50 μL was injected on to ion exchange column (Amino Quant 100 A°, 5 μm, 2.1 × 200 mm, Agilent Technologies, U.S.A). The analysis were done using the Agilent 1100 series RP HPLC system equipped with a thermostat column compartment, binary pump and variable wavelength UV detector. Before estimation of amino acid content (tyrosine) in protein hydrolysates, the system was calibrated with mixture of authentic amino acids and internal standard (norvaline) in the range of 10 pmole/L–10 nmole/L as described in the manufacturers' instruction (Agilent Technologies).

The chromatographic conditions for amino acid analysis were as follows: Mobile phase; A = 20 mM sodium acetate and 0.018% triethylamine pH adjusted to 7.2 with 2% acetic acid. B = 20% of 100 mM sodium acetate pH 7.2 (adjusted with 2% acetic acid) and 40% acetonitrile and 40% methanol. Gradient program used were as follows: 0 to 17 min, 0 to 60% B (0.5 mL/min); 17.1 to 24 min, 60 to 100% B (0.5–0.8 mL/min); 24.1 to 26 min, 0% B (0.5 mL/min) with post run time 5 min. The elution was monitored at 338 nm and the column temperature was maintained at 40°C. Data acquisition was performed using Chemstation Software Version 6.01 (Agilent Technologies U.S.A). The intra assay

rich2/bt-clb/bt-clb/bt-orig/bt5785d03a | destepha | S=4 | 5/1/03 | 4:50 | Art: | Input-1st disk, 2nd MFC/CW-jba

variation in the determination of tyrosine was found to be 0.15 μg/mg protein.

For estimation of 3NTYR, the system was first calibrated with authentic standard of 3NTYR (Sigma Chemical Co., U.S.A) in the range of 5 pmol/L –1 nmole/L. The presence of 3NTYR in the clinical samples was confirmed by spiking with known amount of authentic 3NTYR standard. 50 μL from the aliquot (protein hydrolysate) was directly injected on to RP C18 column (5 μm, 300 A°, 2.1 × 200 mm, Zorbax, U.S.A) and eluted with 100 mM ammonium formate buffer pH 3.5 and acetonitrile. The elution consisted of 16-min linear gradient consisted of 5 to 80% acetonitrile followed by 4 min at 80% acetonitrile (v/v). The flow rate was kept at 0.5 ml/min, with column temperature being maintained at 37°C. Elution was monitored at 365 nm. Under acidic conditions, tyrosine absorbs at 280 nm, while 3NTYR absorbs specifically at 365 nm. The amount of 3NTYR was expressed as μg/mg protein and as a ratio of 3 NTYR/Tyr. The intra assay coefficient for detection of 3 NTYR was found to be 0.012 μg/mg protein.

2.6. SOD assay

Cells were lysed, as mentioned earlier in the text, and the SOD assay was performed following the method of Misra and Firdovich [20]. In brief, epinephrine undergoes auto-oxidation rapidly at pH 10.0 to produce adrenochrome, a pink colored product that was assayed at 480 nm in kinetic mode using UV/VIS spectrophotometer (Beckman DU 640, Beverly, CA, U.S.A). SOD inhibits the auto-oxidation of epinephrine. Standard curve was prepared using purified recombinant human SOD (Sigma Chemical Co., MO, U.S.A.). The rate of inhibition was monitored at 480 nm and the amount of enzyme required to produce 50% inhibition is defined as one unit of enzyme activity. The SOD activity was expressed as units/mg protein. Intra assay variation was found to be 0.35 U/mg protein.

2.7. TBARS determination

TBARS determination in MC was performed by the method described by Devasagayam et. al. with slight modifications [21]. Malondialdehyde (MDA) produced during peroxidation of lipids, serves as an index of lipid peroxidation. MDA, reacts with thiobarbituric acid (TBA) to generate a pink colored product, which was read at 532 nm. Before the assay, fresh calibration graphs were prepared using authentic MDA standard 1,1′,3,3′–tetra ethoxy propane bis (diethyl acetate) and TBA (4,6,dihydroxypyrimidine-2-thiol, Sigma Chemical Co., MO, U.S.A.). The amount of TBARS in PBMC was expressed as nmole of MDA/mg protein. Intra assay coefficient was determined to be 0.55 nmole MDA/mg protein.

2.8. Determination of iron, copper and zinc

MC were digested with 1.0 mL of concentrated nitric acid: perchloric acid (E-Merck, Mumbai, India) mixture (5:1) by heating in china dish for 30 min. The ash was finally dissolved in 2.0 mL of Milli Q water and the amount of iron, copper and zinc were estimated by Atomic Absorption Spectrophotometer (Perkin Elmer 2380, CA, U.S.A.) as described previously [22]. The levels were expressed as μg/mg protein. Intra assay variation for determination of iron, copper and zinc were 0.04, 0.05 and 0.06 μg/mg protein respectively.

2.9. Immunolocalization of iNOS and 3NTYR in ERM of patients with ED

Eight ERM were available for us from patients with ED. The tissues were processed in automated tissue processor (Leica Instruments, Germany) and finally embedded in paraffin wax blocks. 5 μm sections were obtained using a microtome (Leica, JUNG RM 2045, Germany) and the paraffin sections were transferred onto poly L-Lysine coated glass slides. After deparafinization, the sections were incubated with 3% hydrogen peroxide for 10 min to quench the endogenous peroxidase activity. After this step, the sections were gently washed with PBS pH 7.4 (5 min, two changes each) and then exposed to 2% normal rabbit serum (Dako, Copenhangen, Denmark) for 30 min at 4°C.

Sections were again washed with PBS (5 min, two changes each) and then probed with either anti iNOS antibody (rabbit polyclonal, 1: 500, Santa Cruz Biotechnology, CA, U.S.A) or anti 3NTYR antibody (rabbit polyclonal, 1: 750, Cell Signaling Technologies, U.S.A) for 2 h at RT in humid chamber. Following this, the sections were washed in PBS (5 min, two changes each) and then incubated with biotinylated rabbit anti rabbit IgG antibody (1:1000, Dako) for 45 min which was followed by incubation with avidin-biotin complex (Dako) for 15 min at RT. The sections were washed gently with PBS (one minute) and developed using a 3,3′ diaminobenzidine (DAB) substrate kit (Dako).

For histologic correlation, the sections were conterstained in hematoxylin for 45 s, and then mounted (Entellan, Merck, Darmstadt, Germany) and coverslipped. The negative controls for immunohistochemistry included omission of the primary antibody and the use of nonimmune serum in place of the primary antibody. In this case immunoreactivity for iNOS was abolished. However, in case of nitrotyrosine, primary antibody was preadsorbed with 1.5 mM nitrotyrosine for 30 min before immunostaining. This abolished immunoreactivity for nitrotyrosine. Photographic documentation (ASA 100 film, Eastman Kodak, Rochester, NY, U.S.A) were performed with an optical microscope (Nikon, Japan).

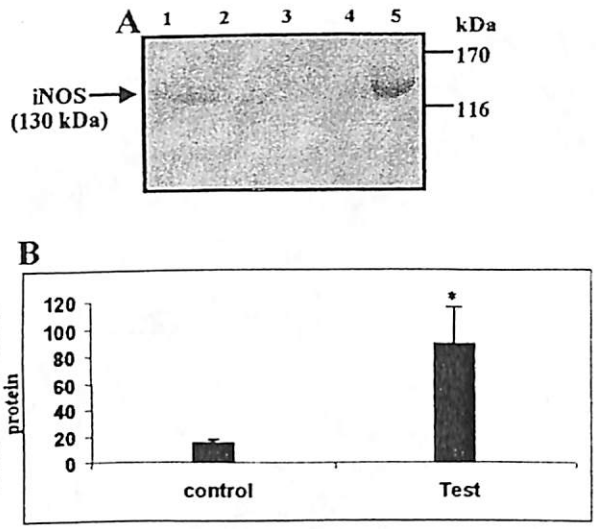

Fig. 1. Western blot analysis of iNOS protein expression in MC. (A) Immunoblot shows the expression of iNOS protein. Lane 1 and 2: Cell lysates from patients with Eales' disease. Lane 3 and 4: cell lysates from healthy control subjects. Lane 5: RAW 264.7 cell line stimulated with lipopolysaccharide (LPS) was used as positive control (B) Quantification of iNOS protein expression in MC of control subjects and patients with Eales' disease. Values are expressed as mean ± SD, $*p < 0.001$.

2.10. Statistical analysis

All values were expressed as mean ± standard deviation. Experimental results were analyzed using a Student's *t*-test. Pearson's correlation test was employed to assess the relationship between the oxidant/antioxidant parameters determined. A P value < 0.05 was considered statistically significant.

3. Results

3.1. iNOS expression

Western blot analysis of iNOS in MC indicated that the expression of iNOS was increased by ~ fivefold, $p < 0.001$ (Figure 1A) in patients with ED when compared with control subjects (Figure 1B).

3.2. NTYR accumulation

Table 4 describes 3NTYR levels in MC of patients with ED and control subjects. Accumulation of 3 NTYR in patients with ED were 2.5fold higher when compared with control subjects ($p < 0.001$).

3.3. SOD activity

The levels of antioxidants and oxidants parameters studied in MC of patients with ED and control subjects are given in (Table 3). MC SOD levels were decreased by twofold ($p < 0.001$) in patients with ED when compared with control subjects (Table 3).

Table 3
Levels of antioxidant and oxidant parameters determined in MC of patients with Eales' disease and control subjects

Parameters	Healthy control subjects (n = 20)	Eales' Disease (n = 35)	*P* value
SOD (Units/mg protein)	7.28 ± 0.31	3.75 ± 0.58	<0.001
TBARS (nmole MDA/mg protein)	11.15 ± 1.67	24.76 ± 2.18	<0.001
Iron*	0.44 ± 0.11	0.72 ± 0.18	<0.001
Copper*	0.38 ± 0.12	0.78 ± 0.21	<0.05
Zinc*	0.62 ± 0.15	0.41 ± 0.09	<0.05

All values are mean ± S.D. *Levels of these trace elements were expressed as µg/mg protein.

3.4. TBARS accumulation

Results indicated that the patients with ED have twofold ($p < 0.001$) increase in the accumulation of TBARS in their PBMC when compared with healthy controls (Table 3).

3.5. Trace elements

We observed 1.7 and 2.0 fold increase ($p < 0.05$) in levels of iron and copper in MC of patients with ED (Table 3). Zinc levels were diminished by 1.5fold ($p < 0.001$) in MC of patients with ED, when compared with healthy volunteers.

3.6. Immunolocalization of iNOS and NTYR

To correlate the changes that we have observed in MC of patients with ED are really consistent with the disease process, we performed immunolocalization experiments on the ERM, which were available to us from the patients with ED. We used this tissue to analyze the immunoreactivity of iNOS expression and NTYR accumulation in leukocyte infiltrates in ERM of patients with ED. We observed strong immunoreactivity of iNOS (Figure 2B) and NTYR (Figure

Table 4
Levels of Tyr and 3 NTYR in PBMC lysates of patients with Eales' disease and control subjects

Parameters	Control Subjects (n = 20)	Eales' Disease (n = 35)	*P* value
Tyr (µg/ml)	16.2 ± 6.6	18.9 ± 6.2	NS
3 NTYR (µg/ml)	0.38 ± 0.14	1.08 ± 0.39	<0.001
Tyr (µg/mg protein)	4.68 ± 2.34	4.2 ± 1.5	NS
3 NTYR (µg/mg protein)	0.10 ± 0.04	0.24 ± 0.10	<0.001
3 NTYR/Tyr	0.021 ± 0.014	0.054 ± 0.03	<0.001

All values are mean ± SD. NS = Not significant.

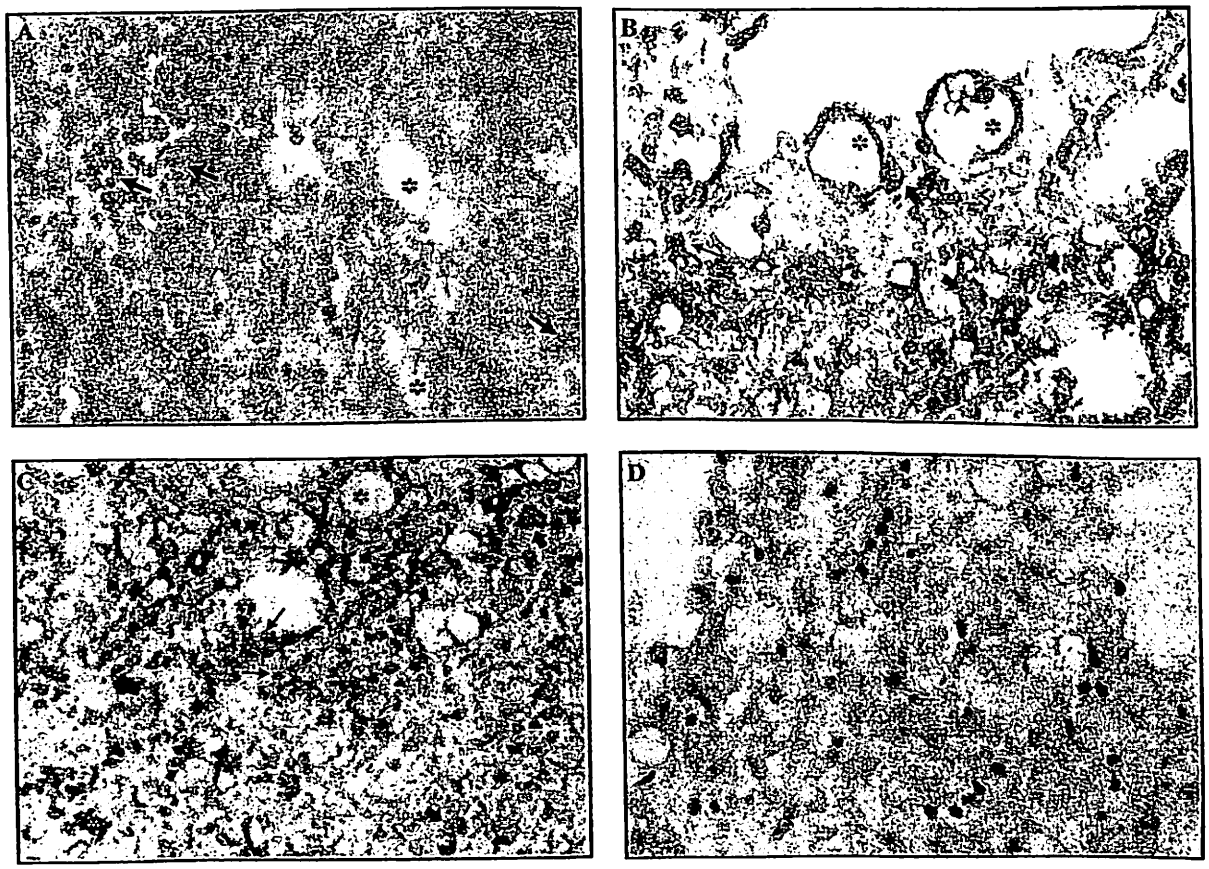

Fig. 2. Immunolocalization of iNOS and 3 NTYR in ERM obtained from patients with ED. (A). Hematoxylin eosin staining of ERM. The Neovascular channels are indicated by bold astrick. Leukocytes are indicated by arrow (×200). (B) iNOS immunolocalization in ERM. Immunoreactivity of iNOS in endothelium is indicated by asterisk, in the lymphocytes by long arrow and monocytes by solid arrow (×400). (C) 3NTYR immunolocalization in ERM. Immunoreactivity of iNOS in endothelium is indicated by astrick. In the lymphocytes by long arrow and monocytes by solid arrow (×200). (D) Negative control (×400). Photographs are representative of eight ERM studied.

2C) in the MC, lymphocytes and in the endothelial cells in all the ERM studied. The nature of inflammatory cells in the ERM were characterized by employing specific CD markers (data not shown).

3.7. Correlation analysis for the variables

Correlation analysis of the variables studied in MC of patients with ED are presented in (Table 5). Increased iNOS expression and 3NTYR accumulation were positively correlated. Increased iNOS expression correlated with diminished SOD activity and increased lipid peroxidation in MC of patients with ED. Elevated levels of iron and copper positively correlated with accumulation of lipid peroxides in MC of patients with ED. Similarly decreased zinc content in MC correlated with diminished SOD activity in patients with ED.

4. Discussion

Oxidative stress has been shown to be involved in the pathophysiology of intra ocular inflammatory diseases [23]. Chronic intraocular inflammation is a major cause of blindness. The loss of vision is the result of damage inflicted by the inflammatory cell infiltration into the retina [23]. The retina is highly prone oxidative damage as it contains large amounts of polyunsaturated fatty acids (PUFA) [24]. RNS and ROS target the PUFA and oxidizes them leading to the formation of lipid peroxides. Therefore to protect the retina from such oxidative stress, the presence of antioxidant en-

Table 5
Correlation between diminished antioxidant levels and accumulation of ROS and RNS in MC of patients with Eales' disease

Variables	r →	P →
iNOS Vs 3 NTYR	0.42	<0.05
3 NTYR Vs SOD	−0.39	<0.05
iNOS Vs SOD	−0.31	<0.05
iNOS Vs TBARS	0.38	<0.05
SOD Vs TBARS	0.39	<0.05
Fe Vs TBARS	0.31	<0.05
Cu Vs TBARS	0.38	<0.05
Zn Vs SOD	0.63	<0.001

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zymes such as SOD, glutathione peroxidase (GPx) catalase, vitamin E and C along with glutathione (GSH) in optimum levels in the cellular milieu is necessary [25]. Our earlier studies revealed diminished SOD and GP_x activity, elevated lipid peroxides and decreased levels of GSH, vitamin E and C in erythrocytes and vitreous of patients with Eales' disease [26,27].

iNOS expression can be triggered by variety of inflammatory mediators, ROS and RNS [28]. The salient aspect of iNOS is that, once activated, it remains active for days and continues to produce NO [28]. iNOS has also got the ability to modulate the acute and chronic inflammatory conditions [29]. This is supported by the findings that NO accumulation is found to be higher in the inflammed tissue [30]. In our present study we have found strong immunoreactivity for iNOS in leukocytes and in the endothelial cells in ERM obtained from patients with ED (Figure 2B). These were consistent with the reports published by other investigators in various inflammatory conditions [reviewed in 31]. Recently Yilmaz *et al.* have reported elevated levels of NO in aqueous humor obtained from patients with Behçet disease (triad of dermatitis, genital ulceration and intraocular inflammation). Increased iNOS expression, diminished SOD activity and NTYR accumulation, have been reported in retinal tissues obtained from animal model of uveitis [32]. In addition to the above, increased lipid peroxide and NO levels were associated with the pathogenesis of age related macular degeneration (ARMD) [33]. In our present study, we have also observed strong relationship between increased iNOS expression and lipid peroxidation in the MC of patients with ED (Table 5).

Superoxide anion ($O_2^{\bullet -}$) reacts with NO to produce peroxynitrite ($ONOO^-$) [34]. The reaction of NO with $O2^{\bullet -}$ is facilitated during inflammatory conditions, since phagocytes have been shown to simultaneously generate NO and $O_2^{\bullet -}$ at a similar rate [35]. $ONOO^-$ is considered a more powerful oxidant than $O2^{\bullet -}$, because the former has the higher diffusion coefficient and half-life than the latter [34]. $ONOO^-$ on entering the cell rapidly nitrates variety of macromolecules chiefly at the aromatic rings [36]. Nitration has been shown to affect the structure and function of various macromolecules, including SOD [37]. This may also be the other reason for diminished SOD activity observed in MC of patients with ED. The diminished SOD activity correlated with increased iNOS expression and NTYR accumulation (Table 5). Zn is considered to be vital component of SOD, which maintains its structural and functional integrity. Zn deficient rats have been shown to have diminished SOD activity and longevity [38]. These effects were reverted, when the animals were supplemented with diet rich in Zn. Considering the above observations, we postulate similar mechanism for observing diminished SOD activity in patients with ED.

Formation of 3NTYR on proteins is considered to be the stable " foot print" of RNS stress [39]. Increased accumulation of 3NTYR has been reported in various pathologic conditions such as uveitis, Alzheimer disease, diabetes mellitus, atherosclerosis plaques, gastric ulcers *etc.*, [reviewed in 39]. Immunolocalization studies for NTYR in ERM of ED, revealed strong immunoreactivity in infiltrating leukocytes and in the endothelial cells, suggesting that our findings were consistent with the disease process. Moreover, we have observed positive correlation between iNOS expression and NTYR accumulation in MC with that of ERM from ED which showed immunoreactivity for iNOS and 3NTYR ($r = 0.914$; $p < 0.001$; $n = 8$).

Diminished SOD activity results in the accumulation of $O_2^{\bullet -}$. $O2^{\bullet -}$ have been shown to release iron and copper from active sites of enzymes involved in respiratory chain [40]. Increased level of Fe and Cu catalyzes the production of hydroxyl radical ($OH^{\bullet}$) *via* Haber-Weiss Fenton reaction [41]. $OH^{\bullet}$ induces lipid, protein and DNA oxidation. These oxidation products have been shown to be elevated in various inflammatory diseases, aging, neurodegenerative diseases, diabetes and other diseases [reviewed in 41]. In this study, we have observed increased iron and copper levels in MC of ED correlating with elevated lipid peroxides in patients with ED.

Both $O_2^{\bullet -}$ and $ONOO^-$ have been shown to be strong chemotatic agents for phagocytes [42]. These reactive species have been shown to involved in initiating, propagating and amplifying tissue damage during inflammatory conditions by their ability to modulate the expression of inflammatory cytokines in the phagocytes [42]. In Behcet's disease, where intraocular inflammation is one of the clinical features, free radical mediated tissue damage has been proposed by many investigators and these reports clearly revealed the role of free radicals in the disease process [43 to 46]. Antioxidant supplementation has been shown to be beneficial delaying clinical symptoms, in diseases were the role of ROS/RNS are associated with the disease process [Reviewed in ref 47]. Recently Köçkam and Naziroğlu have reported the beneficial effect of vitamin E supplementation in delaying the clinical symptoms in patients with Behçet's disease [48]. Likewise Rooji et al. have demonstrated the oral supplementation of vitamin C and E in improving visual acuity in patients with uveitis [49]. In addition to the above, selective inhibition of iNOS expression or supplementation of antioxidant vitamins, has been shown to ameliorate inflammatory tissue damage in uveitis [50].

To conclude, we have reported the potential involvement of ROS and RNS in the development of RV in patients with ED. Considering the potential involvement of free radicals in the disease process, we believe selective inhibition of iNOS and/or supplementation of vitamin C or E might be beneficial to patients with ED in controlling retinal tissue damage.

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