

## Original article

# Expression of oxidative stress in metastatic retinoblastoma- a comparative study

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### Abstract

**Objective:** To compare oxidative stress between primary retinoblastoma and retinoblastoma with distant metastasis.

**Patients and methods:** Forty consecutive patients presented with primary retinoblastoma and the same number of patients presented with distant metastasis, attending the outpatient department of our hospital between August 2002 and April 2005. All the patients with retinoblastoma underwent a standard metastasis workup and were subsequently categorized into two groups (without metastasis and with metastasis). Venous blood samples were drawn from each patient. After proper centrifugation, serum was collected and antioxidant enzymes and reactive oxygen species (ROS) were assayed.

**Main outcome measures:** Serum collected from the patients was subjected to biochemical assay of the antioxidant enzymes (superoxide dismutase, catalase and peroxidase) and ROS to determine any difference in enzyme activity between the two groups.

**Results:** Antioxidant levels were found to be less in the metastasis group as compared to the primary intraocular retinoblastoma group ( $p < 0.05$ ). Mean ROS activity was found to be increased in metastatic group ( $p < 0.05$ ).

**Conclusion:** The decreased antioxidant enzymes level along with increased ROS activity in patients with metastatic retinoblastoma reflect increased oxidative stress as compared to primary intraocular retinoblastoma patients.

**Key-words:** Retinoblastoma, antioxidant enzymes, reactive oxidant species, oxidative stress

### Introduction

Oxidants are chemical products that help in oxidation. Oxidative damage to a cell may lead to loss of barrier function, which in turn will lead to oedema, electrolyte imbalance and eventually, cellular dysfunction. All of them will eventually lead

to cellular dysfunction (Tolbert, 1981). Oxidants are not only implicated in the pathogenesis of chronic diseases like malignancy, age related macular degeneration and cataracts, but also in a variety of acute conditions like acute myocardial infarction, acute renal failure, adult respiratory distress syndrome and even in acquired immunodeficiency syndrome (AIDS).

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Oxidant burden in the tissue can be measured by electron spin resonance spectroscopy, gas-liquid chromatography, mass spectrometry, high performance liquid chromatography and other enzymatic and immuno-reactive procedures. Additionally, by estimating the levels of antioxidants such as superoxide dismutase, catalase and peroxidase in serum, cell culture and tissue mass, we can assess levels of oxidative stress. Oxidative stress in the human body is a delicate balance between activities of antioxidant enzymes and reactive oxidant species (ROS). We conducted this study to compare the burden of oxidative stress as reflected in the sera of two groups of retinoblastoma patients.

### Patients and methods

In this study, 80 consecutive patients with a clinical diagnosis of retinoblastoma who presented at the pediatric and general ophthalmology outpatient departments of our institute between August 2002 and April 2005 were recruited for the study. Each of the patients underwent standard examination protocol as described below. Based on clinical and investigational data, the total patient pool was divided into two groups:

Group A: Patients with primary intraocular retinoblastoma

Group B: Retinoblastoma with metastasis

Blood samples were collected from each of the patients and estimation of three antioxidant enzymes (superoxide dismutase, catalase and peroxidase) and ROS were performed at the Department of Biochemistry, Calcutta University.

Clinical and investigational workup included: 1. Complete ocular examination including indirect ophthalmoscopy, 2. Routine complete haemogram, stool examination, 3. CT scan of brain and orbit 4. Whole abdomen Ultrasonography, 5. Chest X-ray, 6. CSF cytology in selected cases, and 7. Serum assay of superoxide dismutase, catalase, peroxidase and ROS.

Informed consent was taken from the legal guardian of each patient. Institutional ethical committee approval was obtained before recruitment of the first patient.

**Collection of blood:** Using a disposable syringe, 2-3 ml of blood was collected by venepuncture in plastic tubes without any anticoagulant. The tubes were centrifuged for 10 minutes at 2800 rpm. Supernatant serum was pipetted out with a micropipette, transferred to an Eppendorf tube, and stored in deep freeze. Biochemical estimation was performed from these sera in the following methods.

**Estimation of superoxide dismutase:** Superoxide dismutase activity was measured by the method employed by Marklund and Marklund (1974).

Reagents:

- (i) Tris cacodylate buffer (50 mM, pH 8.2 with 1 mM diethylene triamine pentaacetic acid) 20 ml of 50 mM tris (mol wt 121.14) and 8 ml of 50 mM cacodylic acid (mol wt 137.88) gave a pH of 8.2.
- (ii) Pyrogallol solution: Dissolve 0.00757 grams of pyrogallol in 20 mL water.

Procedure: 10 – 50 mL of serum was taken and buffer was added to make a total volume of 0.95 mL in 12 x 100 mm tubes. This was mixed thoroughly. The solutions were incubated at room temperature for 1 hour during which time the increasing optic density (OD) due to turbidity reached a steady state in all samples. 0.05 mL of 4 mM pyrogallol solution was added, mixed 6 times, and the change in O.D. was recorded at 420 nm at room temperature in order to attain 60-80% inhibition of pyrogallol auto-oxidation in alkaline solution (pH 8.2).

**Catalase estimation:** Catalase activity was assayed by measuring the rate of breakdown of H<sub>2</sub>O<sub>2</sub> at 240 nm according to the method of Luck (1965).

1 mL reaction mixture contained 0.05 M potassium phosphate buffer (pH 7), 11.6 mM H<sub>2</sub>O<sub>2</sub> and 50 mL of enzyme. Catalase activity was calculated

based on an extinction coefficient  $43.1 \text{ M}^{-1} \text{ cm}^{-1}$  for  $\text{H}_2\text{O}_2$  at 240nm and expressed as nmols of  $\text{H}_2\text{O}_2$  consumed/min/mg of protein.

Buffer	$\text{H}_2\text{O}_2$	Enzyme
1.9	1 ml	100 mL

**Peroxidase assay:** Peroxidase activity was estimated according to Chen and Asada (1989). The assay mixture (3mL) contained 50 mM potassium phosphate buffer (pH6), 20 mM O-dianisidine, 0.1 mM  $\text{H}_2\text{O}_2$  and 10 $\mu\text{L}$  of enzyme extract. The peroxidase activity was calculated using an extinction coefficient of O- dianisidine ( $11.3 \text{m M}^{-1} \text{ cm}^{-1}$ ) at 460nm and expressed as  $\mu\text{mol}$  O-dianisidine oxidized /min/mg of protein.

Buffer/ $\text{H}_2\text{O}_2$	O-dianisidine	Enzyme
2.9	50 $\mu\text{L}$	50 $\mu\text{L}$

**Estimation of ROS:** The ROS activity was measured using the method employed by Aitken (1992). 400 $\mu\text{L}$  of sera was mixed with luminal (5-amino-2,3-dihydro-1,4-phthalazinedione) prepared as a 25mM stock in DMSO (Dimethyl sulphoxide) together with 8 $\mu\text{L}$  of horse-radish peroxide. Levels of ROS were determined by measuring chemiluminescence for 15 minutes and results were expressed in counted photons per minute (c.p.m).

Data obtained were documented and analysed. The Student's t-test was used to ascertain the level of significance between two groups. A p-value of less than 0.05 was considered statistically significant.

## Results

Out of total 40 cases of primary intraocular retinoblastoma 25 are male patients (62.5%) and 15 are female patients (37.5%). The sex ratio is 1.67. Slight male predominance in this study is probably due to selection bias. According to Apple and Rabb (2000), no sexual predilection has been reported. Most numbers of cases (total 19, 23%) belong to 1.5 to 2-year age group.

The mean serum superoxide dismutase level in cases with metastatic retinoblastoma was 25.232 (SEM=

$\pm 0.527$ ), whereas the same in the primary intraocular retinoblastoma group was 16.638 (SEM=  $\pm 0.633$ ). Since these data were in terms of unit of O.D. change at 420 nm per 50% inhibition of pyrogallol auto-oxidation in alkaline media, higher the value lower was the serum SOD activity. So it was readily apparent that in each of the cases value of serum superoxide dismutase was lower in patients of metastatic retinoblastoma when compared to the same level in intraocular group [ $p < 0.05$ ].

The mean serum catalase level in cases with metastatic retinoblastoma was 0.516 (SEM=  $\pm 0.0077$ ). The mean serum catalase level in the other group was 0.623 (SEM=  $\pm 0.0046$ ). It was apparent that the mean serum catalase level in patients of metastatic retinoblastoma was lower than the same level in primary group [ $p < 0.05$ ].

The mean serum peroxidase level in primary group was 0.145 and the standard error of mean was  $\pm 0.0030$ . The mean serum peroxidase level in cases with metastatic retinoblastoma was 0.115 and the standard error of mean was  $\pm 0.0018$  ( $p < 0.05$ ).

Mean serum ROS activity in metastatic group was  $4344662 \pm 2886889$  and the same activity in primary tumor group was  $1832010 \pm 1798019$  ( $p < 0.05$ ).

## Discussion

A free radical is any species that contains one or more unpaired electrons (Halliwell 1989). Though some oxidizing agents such as hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), hypochlorous acid (HOCl) are not radicals, they still can become involved in free radical reactions. The term reactive oxidant species (ROS) includes not only the oxygen radicals ( $\text{O}_2$ , OH), but also the compounds such as  $\text{H}_2\text{O}_2$  and HOCl (Southorn, 1988). Antioxidants are compounds that remove or prevent the formation of reactive oxygen species as therapeutic agents (Curnutte and Baboir, 1987). When generated at a low (physiological) level, ROS can perform useful functions in human body, but at a higher concentration, they are cytotoxic (Maly 1990).

If a radical reacts with a non-radical, another free radical is produced. This property enables free radicals to participate in chain reactions. Radicals may also serve as oxidants and reductants. Peroxidation of membrane lipid produces malonaldehyde as a by-product. As previously mentioned, the conditions such as photochemical reactions, radiation injury, drugs, hypoxia, inflammatory conditions, viral infections (including H.I.V), and carcinogenic process produce abundant reactive oxygen species in human body resulting in a state of oxidative stress.

Our body has antioxidant defence mechanisms to handle this oxidative burden. Hence, direct and indirect evidence of oxidant stress may be ascertained by either detection of radicals themselves or by detection of biological damage caused by these radicals (like malonaldehyde, thymine glycol, 5-hydroxymethyluracil etc.) or by assessing the antioxidant defence mechanism (like SOD, catalase, peroxidase, ceruloplasmin and transferring).

Antioxidants are the chemical products nullifying the effects of oxidants in the body. Depending upon their location, they are classified into extracellular and intracellular components. The major extracellular antioxidant components are ceruloplasmin, uric acid, glucose, alpha tocopherol and albumin.

Most of the intracellular defence mechanisms in the body are in the form of enzymes like superoxide dismutase, peroxidase and catalase. Superoxide dismutase protects cells against reactive free radicals produced by ionizing radiation or other mechanisms, such as carcinogenesis. This enzyme catalyzes the dismutation of the superoxide anion into  $O_2$  and  $H_2O_2$  (Weisiger and Fridovich, 1973). There are three types of SOD found in mammals. CuZn-SOD is present in cytosol (Tyler 1975). Mn-SOD is present in mitochondrial matrix (Southorn and Powis, 1988).

Two antioxidant enzymes in the body, glutathione peroxidase and catalase mediate the catalytic

breakdown of hydrogen peroxide. These two enzymes act at two different concentrations of hydrogen peroxide in the body. At low hydrogen peroxide concentration, the role of enzyme glutathioneperoxidase (GPO) is activated (Paller et al,1984). At high concentration of hydrogen peroxide, the enzyme catalase is activated to remove it (Salim 1993).

Malignant conversion of a normal cell occurs in three stages - initiation, promotion and progression. Oxidation may involved in all the three stages of carcinogenesis process. Oxidants may produce genetic lesions, which are toxic to the cell itself. Rearrangement of promoter regions, deletions and inactivation or loss of tumor suppressor gene may lead to initiation of the carcinogenic process. In retinoblastoma, tumor suppressor gene is inactivated by structural alteration of a vital base pair, commonly C-G (Cytosine-Guanine) in retinoblastoma. When cellular genes are converted to oncogenes, G-C and A-T base pairs present two vulnerable targets for attack by oxidants. Copper ion bound to DNA may augment the oxidative DNA damage. Oxidative stress may also produce 5hydroxy cytosine. Thymine glycol is toxic to DNA if not excised by a DNA-glycosylate prior to replication. The cleavage of this base can give rise to abnormal sites, which in turn may enhance the mutagenesis (Albeno et al, 1991).

Out of total 40 cases of primary intraocular retinoblastoma 25 are male patients (62.5%) and 15 are female patients (37.5%). The sex ratio is 1.67. Slight male predominance in this study is probably due to selection bias. According to Apple and Rabb (2000), no sexual predilection has been reported. Most numbers of cases (total 19, 23%) belong to 1.5 to 2-year age group. This was supported by Abramson (1985).

In all 40 cases of metastatic retinoblastoma the serum SOD level was low when compared to the primary intraocular retinoblastoma group [ $p<0.05$ ]. This finding tallies with that of Oberley (1979) and Weis (1980), who found a low CuZnSOD activity

in tumor cells as well as a low MnSOD level in malignant cell-lines. In the metastatic retinoblastoma group, serum peroxidase and catalase activity is lower than those in the primary intraocular retinoblastoma group [ $p < 0.05$ ]. These observations are identical to the findings reported by Batko et al (1996) who found a low catalase activity in neoplastic tissue. Asano et al (1985) also reported an identical reduction of catalase activity in carcinomatous tissues. However, the findings of the present study did not tally with that produced by Asano et al (1985) who reported a higher peroxidase level in tumor tissues.

Cytotoxicity and growth stimulation are intimately related in oxidant induced carcinogenesis process. By stimulating terminal differentiation, promoters may induce compensatory proliferation. Here, the initiated cells proliferate to fill the void left by the removal of their normal counterpart. Gupta et al (1984) and Vallyathan (1998) demonstrated increased ROS activity associated with carcinogenesis. Cellular defences may alter the level of toxicity caused by ROS and play a preventative role against carcinogenesis, according to Kemsler et al (1984) and Oberley et al (1997).

### Conclusion

Our study highlights the increased level of oxidative stress in patients with the metastatic retinoblastoma. Increased ROS activity coupled with decreased level of antioxidant enzymes support the oxidative stress mediated injury to cellular structures in these patients.

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