Absence of abnormal erythrocyte superoxide dismutase, copper, or zinc levels in patients with retinitis pigmentosa

ROSALIE K. CROUCH AND JERRE K. CHAMBERS

From the Department of Ophthalmology, Storm Eye Institute, Medical University of South Carolina, Charleston, South Carolina 29425, USA

SUMMARY Blood samples obtained from 22 patients with retinitis pigmentosa, 6 unaffected family members, and 8 unrelated controls showed serum copper and zinc to be in the normal range, contrary to the results in earlier reports. Likewise no significant variation of erythrocyte superoxide dismutase (SOD) and catalase levels was found between the 3 groups or when the patients were grouped by sex, age, or genetic distribution. The SOD proteins from controls and retinitis pigmentosa patients had identical mobilities on gel electrophoresis, isoelectric points, and heat stabilities. Our studies do not support the use of copper, zinc, or SOD in the diagnosis or treatment of retinitis pigmentosa.

Several studies have considered the connection between possible abnormalities in serum copper levels and the disease retinitis pigmentosa (RP). Gahlot *et al.*¹ reported that for 15 patients serum copper levels were within a normal range but the plasma ceruloplasmin was decreased. In contrast Rao *et al.*² reported an elevated serum copper level in a series of 24 patients with decreased ceruloplasmin levels. Bastek *et al.*³ also reported elevated copper levels in a family with X-linked RP. These results all imply an abnormal copper metabolism in RP patients. However, Ehlers and Bülow⁴ found normal values for both serum copper and plasma ceruloplasmin in a series of 11 RP patients. Marmor *et al.*⁵ likewise found no abnormalities in serum copper, serum ceruloplasmin, and urinary copper excretion in 38 patients and 15 family members with recessive, dominant, and X-linked forms of RP.

About 60% of total red cell copper occurs incorporated in the enzyme superoxide dismutase (SOD, EC 1.15.1.1),⁶ which contains both copper and zinc. Copper is essential for the activity of the enzyme.⁷ Nutritional experiments in animals have confirmed that deficiency in copper can decrease the SOD activity in several tissues.⁸⁻¹⁰ In a person with copper deficiency the erythrocyte SOD is reported to be

decreased by about 50%.¹¹ Furthermore, measurements with radioactive copper suggest that erythrocyte SOD obtains its copper from ceruloplasmin, indicating that a decrease in ceruloplasmin might affect SOD activity.¹²

The retina has been shown to contain several SOD isoenzymes, the major one being the same as the erythrocyte Cu-Zn enzyme.¹³ SOD is part of the natural defence mechanism against oxygen damage and acts as a scavenger for the toxic superoxide radical.¹⁴ Any decrease in the level of this enzyme could result in the accumulation of the toxic superoxide radical which has been implicated in lipid peroxidation, sulphhydryl group oxidation, and DNA damage.

The toxicity of the superoxide radicals can also be produced by their reaction with hydrogen peroxide, generating hydroxyl radicals which are highly reactive and affect most biological materials.¹⁵ The enzyme catalase inhibits this reaction by eliminating hydrogen peroxide. The damaging effects of a decreased SOD level might be overcome by an enhanced catalase content, and so this enzyme is also of interest. In view of the conflicting data on copper levels in RP patients and the biological importance of SOD we have compared the levels of copper, zinc, catalase, and SOD in the blood of RP patients, normal relatives,

Correspondence to Dr Rosalie K. Crouch.

and controls. We have also studied the electrophoretic and heat sensitivity properties of the erythrocyte SOD in an attempt to detect any abnormalities in the enzyme obtained from RP patients.

Materials and methods

Venous blood was drawn from volunteer patients (8 males and 14 females between the ages of 14 and 66), 6 family members, and 8 healthy controls. Informed consent was obtained from each volunteer after the nature of the procedure had been explained. All the patients had been diagnosed as having RP by an ophthalmologist. A family history was taken from each patient. The majority of the patients had no family history of the disease, and these diseases were grouped as simplex. The disease in the remainder of the patients was found to be recessive, X-linked, or dominant. Heparinised blood (1000 units/ml) was used for the haemoglobin, haematocrit, catalase, and SOD assays. Serum was used for the copper and zinc assays. Enzyme and haematocrit measurements were conducted on the same day the bloods were drawn, with control and test samples being processed simultaneously. The haemoglobin, copper, and zinc assays were performed on samples frozen at -70°C for up to one week.

The haematocrit was measured with a Sedinak haemometer. The haemoglobin concentration was determined by Drabkin's reagent¹⁶ (Sigma). Catalase activity was measured colorimetrically with dichromate in acetic acid as described by Sinha.¹⁷ Activity of catalase is expressed as μmol hydrogen peroxide decomposed per min per mg of haemoglobin. The haemoglobin concentration was adjusted to 0.1 g/ml and haemoglobin-free samples were prepared for the SOD assay as described by Winterbourn *et al.*¹⁸ The SOD activity was assayed by inhibition of nitroblue tetrazolium reduction by superoxide radicals generated by the xanthine-xanthine-oxidase system.¹⁹ SOD activity is expressed as units/mg of haemoglobin where one unit (U) of activity is defined as the amount of enzyme activity which inhibits the rate of reduction of nitroblue tetrazolium by 50%. It should be noted that the SOD activity values from different laboratories vary widely. The normal values obtained in this laboratory are in the range of 2.0–3.5 U/mg haemoglobin, which agree with those of Winterbourn *et al.*¹⁸ Copper and zinc were determined by atomic absorption spectrometry.²⁰ Triplicate measurements were obtained on each sample for all assays.

Data were treated statistically by Student's *t* test.²¹ The variability of the data is presented as mean $\pm$ SEM. Differences at $p < 0.05$ were considered significant.

Disc electrophoresis was performed in 7.5% polyacrylamide gels with the system described by Maurer²² except that stacking gels were not employed. Isoelectric focusing was performed in 7.5% polyacrylamide gels with a final concentration of 5% ampholytes (v/v) in the ranges of pH 3.0–10 and pH 4–6.5 (Pharmacia Fine Chemicals).²³ The potential was maintained at 200 V for 3.5 h. After focusing a blank gel was sliced into sequential 5 mm sections. The sections were incubated overnight in 2 ml water and the pH determined. The gels were stained for SOD activity at 37°C for 1 hour as previously reported.¹³ Absence of dye uptake indicated reaction of the stain with the enzyme and presence of the enzyme. Duplicate gels were stained for protein with Coomassie brilliant blue according to the method of Weber and Osborn.²⁴

Heat sensitivity was determined by heating a haemoglobin-free sample¹⁸ to a specific temperature and removing aliquots at certain time intervals. The aliquots were assayed immediately. The samples from control and RP patients were matched for age and sex.

Results

A comparison of copper, zinc, catalase, and SOD levels between 22 RP patients, 6 unaffected family members, and 8 unrelated controls showed all values to be within normal ranges. No significant differences were observed for any of metals or enzymes tested (Table 1). The haematocrits were determined and also found to be within the normal range of 36 to 52 for all 3 groups. The ages of the RP patients studied varied from 14 to 66, but again when comparisons with controls were made by age and sex no significant differences were found (Fig. 1, Table 2).

Recessive, dominant, and X-linked forms of RP were represented in this study, but the majority of our patients had no family history of the disease (simplex). A similar genetic distribution has been

Table 1 SOD, catalase, copper, and zinc levels in RP patients, family members, and controls

	Cu $\mu\text{g}/100\text{ ml}$	Zn $\mu\text{g}/100\text{ ml}$	Catalase* U/mg H	SOD U/mg H
RP patients (n=22)	100 $\pm$ 13	104 $\pm$ 15	5.9 $\pm$ 0.9	2.6 $\pm$ 0.7
Family members (n=6)	115 $\pm$ 10	102 $\pm$ 6	5.1 $\pm$ 1.2	3.4 $\pm$ 0.8
Controls (n=8)	107 $\pm$ 9	108 $\pm$ 9	5.8 $\pm$ 1.4	3.2 $\pm$ 0.7

* $\mu\text{moles H}_2\text{O}_2$ decomposed per min per mg H.

H=Haemoglobin.

Each value given as the mean $\pm$ SEM; for $n \geq 2$ the SEM is not applicable.

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Fig. 1 Copper, zinc, SOD, and catalase levels compared by age: RP patients; unaffected family members; unrelated controls. Brackets represent SEM; n=number of samples in group; H=haemoglobin.

Table 2 Male and female: copper, zinc, SOD, and catalase levels

Group	Sex	n	Cu $\mu\text{g}/100\text{ ml}$	Zn $\mu\text{g}/100\text{ ml}$	Catalase* U/mg H	SOD U/mg H
RP patients	Male	8	107 $\pm$ 15	108 $\pm$ 15	6.1 $\pm$ 0.4	2.9 $\pm$ 0.5
	Female	14	94 $\pm$ 13	95 $\pm$ 10	5.6 $\pm$ 0.8	2.5 $\pm$ 0.6
Family members	Male	1	120	110	5.4	3.6
	Female	5	112 $\pm$ 8	98 $\pm$ 1	5.0 $\pm$ 1.0	3.3 $\pm$ 0.5
Controls	Male	4	110 $\pm$ 10	112 $\pm$ 10	5.5 $\pm$ 1.0	2.8 $\pm$ 0.8
	Female	4	104 $\pm$ 7	103 $\pm$ 12	6.2 $\pm$ 0.9	3.3 $\pm$ 0.9

* $\mu\text{moles H}_2\text{O}_2$ decomposed per min per mg H.

H=Haemoglobin.

Each value is the mean $\pm$ SEM; for n $\geq$ 2 the SEM is not applicable.

reported by Berson *et al.*²⁵ From family history the RP patients were grouped as shown in Table 3, and again there was no significant variation of SOD levels.

The erythrocyte SOD enzymes were examined by

Fig. 2 Polyacrylamide gel isoelectrophoretic focusing patterns. Normal and RP haemolysates: (a) Gels stained for protein: 1—RP; 2—control. (b) Gels stained for SOD activity: 1—RP; 2—control; absence of stain indicated SOD activity. The pH range of the ampholytes was 3-10.

gel electrophoresis to determine if there were any major differences between controls and RP patients in the enzyme molecular weight or charge distribution. On 7.5% polyacrylamide gels stained for both protein and SOD activity no differences were

Table 3 Inheritance patterns of copper, zinc, catalase, and SOD levels in retinitis pigmentosa patients

	n	Cu µg/100 ml	Zn µg/100 ml	Catalase* U/mg H	SOD U/mg H
Simplex	12	104±10	106±12	5.7±1.0	2.8±0.5
Recessive	7	98±11	101±5	6.0±0.5	2.9±0.9
Dominant	2	103	98	5.5	2.2
X-linked	1	92	105	5.5	2.6
Female carrier	1	115	104	5.9	3.0

*µmoles H₂O₂ decomposed per min per mg H.
H=Haemoglobin.
Each value is the mean ± SEM; for n≥2 the SEM is not applicable.

observed between control and RP samples. Likewise the isoelectric focusing profiles (Fig. 2) showed the protein with major SOD activity to be identical for both groups with a pI of 4.75, which is identical to that reported for the human SOD.²⁶

A comparison of the sensitivity to heat of the SOD enzyme from control and RP patients was made (Fig. 3), and no significant variation in the enzyme stability was observed. At 72°C enzymes from both groups lost about 90% activity in 20 minutes.

Fig. 3 Temperature inactivation of enzymes from RP patients and controls. Haemoglobin-free enzyme solutions were subjected to heat at 65°C and 72°C. Aliquots were withdrawn at times indicated and the activities assayed. O—O controls; X—X RP samples.

Discussion

Abnormalities in both copper and zinc levels have been reported in patients with RP.^{1,2} As the major erythrocyte and retinal SOD enzyme contains both copper and zinc, and as copper deficiency has been linked to a SOD decrease, it seems logical that there might be an abnormal level of this enzyme in RP patients. However, the results of this study did not support these earlier reports of abnormal copper or zinc levels in RP patients. The data were grouped according to age, sex, and inheritance patterns, but no significant variation was detected in any of these classifications. Particular attention was paid to the one case of X-linked RP which we had in the study, but contrary to the report by Bastek *et al.*² we did not find elevated copper levels in this patient or the carrier, nor did we find a higher level of zinc in this female carrier. Therefore from our data, and in agreement with the studies of Marmor *et al.*⁵ and Ehlers and Bülow,⁴ we found no evidence of abnormal copper and zinc metabolism in RP patients, at least in this patient population.

As the copper and zinc levels appear to be normal in RP patients, it is not surprising that we did not detect any abnormalities in SOD levels. Additional evidence that there are no alterations in the SOD enzyme itself is gained from the electrophoretic and heat stability properties of enzymes from control and RP patients. The identical gel patterns of the samples show that the molecular weight and charge distribution are the same for the enzymes. A decrease in the heat stability of several enzymes including SOD²⁷ with age has been observed. However, in samples taken from controls and RP patients of similar age and sex the sensitivity to heat was the same, and thus there is no premature 'aging' or inactivation of the enzyme. Likewise we observed no major abnormalities in catalase levels. It should be noted that the derivations of the catalase measurements are high and only major differences would be noted. Thus the erythrocyte levels of these enzymes protecting against oxygen toxicity are within the normal range for patients with RP.

The reasons for the discrepancies between our results on copper and zinc levels and those of earlier reports^{1,3} remain to be explained and may be due to differences in patient population. However, our studies do not support the use of copper, zinc, or SOD in the diagnosis or treatment of RP.

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