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Original Article

Serum superoxide dismutase activity in acute and chronic paediatric liver diseases

Mortada Hassan El-Shabrawi [a], Naglaa Mohamed Kamal [a,*], Fawzi A. Halawa [b], Mohamed A. El-Guindi [c], Gihan Ahmed Sobhy [c]

[a] Paediatric Hepatology Unit, Faculty of Medicine, Cairo University, Cairo, Egypt
[b] Biochemistry Department, Faculty of Medicine, Cairo University, Cairo, Egypt
[c] Paediatric Department, National Liver Institute, Menoufya University, Menoufya, Egypt

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ABSTRACT

Background and study aims: Measuring serum superoxide dismutase (SOD) levels in infants and children having acute or chronic liver disease of different aetiologies, and correlating these levels with disease aetiology in an attempt to clarify the role of SOD as an antioxidant in these diseases.

Patients and methods: We prospectively enrolled 58 infants and children and divided them into four groups: Group I, 24 patients with surgical cholestasis; group II, 11 patients with medical cholestasis; group III, nine patients with autoimmune chronic hepatitis; and group IV, 14 patients with viral hepatitis. Forty healthy age- and sex-matched children served as controls. Serum SOD activity was measured in all patients and controls using spectrophotometry.

Results: The level of SOD showed a statistically significant increase in patients with medical cholestasis compared to healthy controls ($p < 0.0001$). SOD activity of other groups showed no significant difference compared to controls.

Conclusions: Significantly increased serum SOD in infants and children with medical cholestasis is probably consequent to its increase in liver tissue in response to the liberation of reactive oxygen species. This suggests that products of free radical reactions might be involved in the pathogenesis and/or progression of medical cholestasis, and that SOD might attempt to minimise the liver injury.

Introduction

Reactive oxygen species (ROS) have both hazardous and beneficial roles. Nitric oxide synthase is one of the enzymes responsible for their generation. Their beneficial effects occur at low-to-moderate concentrations in cellular defence mechanisms against infectious agents, in the function of a number of cellular signalling pathways, and the induction of a mitogenic response. By contrast, excess production of ROS results in oxidative stress [1].

Oxidative stress happens when production of oxygen radicals inside body cells exceeds their antioxidant capacity leading to damage of cells' essential macromolecules with the resultant abnormal gene expression, disturbance in receptor activity, proliferation or cell death, immunity perturbation, mutagenesis, protein or lipofuscin deposition [2]. Deposition of ROS is the function of antioxidant enzymes, mainly, superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) [3]. SOD is a key component in the free radical detoxification process. It is a metalloenzyme which catalyses the dismutation of superoxide radical into oxygen and hydrogen peroxide. Its function appears to be protection of cells from the toxic effects of the endogenously generated superoxide radicals [4]. Eukaryotic cells have an extracellular SOD and two forms of intracellular SOD: one is found in the mitochondrial matrix, the manganese SOD (Mn-SOD), and another is predominantly present in citosol, the copper–zinc SOD (CuZn-SOD).

Synthesis of CuZn-SOD is constitutive, whereas Mn-SOD expression is enhanced by different factors such as inflammatory mediator agents causing oxidative insult and hyperoxia which suggest together with the strategic localisation of Mn-SOD in mitochondria, which is the main site of ROS production, that Mn-SOD is an important protective agent against oxidative stress [5].

Disturbances in the antioxidant system could play a role in the pathogenesis of acute and chronic liver disease [6,7]. Liver disease in infancy has multiple aetiologies. As reactive oxygen

* Corresponding author. Tel.: +20 1141991114.
E-mail addresses: Nagla.kamal@kasralainy.edu.eg, Kamaln30@yahoo.com (N.M. Kamal).

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intermediates are involved in several types of tissue damage, it has been investigated whether different forms of liver disease in infancy are associated with increased free radical generation, using an indirect approach in which SOD (a free radical scavenger) activity is determined in the liver tissue [5,8,9], erythrocytes [4], peripheral blood monocytes (PBMCs) [5] or in the serum [9–13].

The aim of this study is to indirectly evaluate the oxidative status in paediatric liver diseases of various aetiologies, whether acute or chronic by measuring the antioxidant enzyme SOD in serum.

Patients and methods

We prospectively enrolled 58 infants and children with various acute and chronic liver diseases from June 2011 to November 2012 from those attending the Paediatric Hepatology Unit, Cairo University Children Hospital, Cairo, Egypt. Forty age- and sex-matched, healthy infants and children comprised the control group. The Institutional Review Board (IRB) of our hospital approved the study and informed, written consents were obtained from parents of all enrolled children.

Patients with acute and chronic liver diseases were divided according to the aetiology of liver disease into four groups:

- Group I: This group included 24 patients with surgical cholestasis, 14 females and 10 males (22 had biliary atresia and two had a choledochal cyst).
- Group II: This group included 11 patients with medical cholestasis, seven females and four males (six had neonatal hepatitis (NH), three had cytomegalovirus (CMV) hepatitis and two had paucity of interlobular bile ducts (PIBDs)).
- Group III: This group included nine patients with chronic autoimmune hepatitis (AIH), seven females and two males.
- Group IV: This group included 14 patients with viral hepatitis, four females and 10 males.

The control subjects were 23 females and 17 males.

All patients and control subjects were subjected to full history taking, thorough clinical examination, laboratory investigations including urine and stool analysis, complete blood count, biochemical tests of liver function (total and direct serum bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), total serum proteins and albumin, prothrombin time (PT) and international normalised ratio (INR) of prothrombin) as well as abdominal ultrasonography. Viral hepatitis markers, immunoglobulin profile, autoantibodies, hepatic iminodiacetic acid (HIDA) radionuclide liver scan and liver biopsy were performed as indicated.

SOD assay

Blood was extracted, centrifuged and SOD activity was measured in serum by a spectrophotometric assay using the BIOXYTECH SOD-525 assay (Oxis International, Inc., Portland, OR, USA) based on the SOD-mediated increase in the rate of autoxidation of 5,6,6a,11b-tetrahydro-3,9,10-tri-hydroxy-benzo[c]fluorene R1 in aqueous alkaline solution to yield a chromophore with maximum absorbance at 525 nm [14]. Interference due to mercaptans is controlled by pre-treating samples with 1,4,6-trimethyle-2-vinyl-pyridinium R2.

The SOD activity is determined from the ratio of the autoxidation rates in the presence (*Vs*) and in the absence (*Vc*) of SOD. The *Vs*/*Vc* ratio as a function of SOD activity is independent of the type of SOD being measured [11]. One SOD-525 activity unit is defined as the activity that doubles the autoxidation background (*Vs*/*Vc* = 2).

Sample preparation

(1) A 1.5-ml blood sample was collected from each patient and control in heparinised tubes.
(2) The whole blood was centrifuged at 2500*g* at 40 °C for 5 min and supernatants plasma collected.
(3) Ice-cold reagent, 400 ml (absolute ethanol/chloroform, 62.5/37.5 (v/v)), was added to 250 μl of plasma in glass test tubes.
(4) Vortex for at least 30 s and centrifuge at 3000*g* at 40 °C for 10 min.
(5) Collect the upper aqueous layer and keep at 0–40 °C for the assay.

Assay procedure

- *Set-up:* (1) Place the required amount of buffer at 37 °C in an open container and (2) calibrate the spectrophotometer at 525 ± 2 nm against air.
- *Assay of SOD activity:* (1) Add 900 μl buffer to a test tube for each blank or sample; (2) add 40 μl blank or sample to the test tube; (3) add 30 μl of R2 to the test tube and vortex; (4) incubate at 37 °C for 1 min; (5) add 30 μl of R1 to the test tube and vortex briefly; and (6) immediately transfer to a spectrophotometric cuvette and measure the absorbance over time.

Calculations

Let *Vc* and *Vs* be the reaction rates of the control and the sample, respectively. The SOD activity of each sample can be obtained from the experimental *Vs*/*Vc* ratio, through inspection of a reference table showing the relationship between the experimental *Vs*/*Vc* ratio and the SOD activity. The relationship of the relative rate (*Vs*/*Vc*) to SOD-525 activity (U-525) is described by the following equation:

$Vs/Vc = 1 + (\mathrm{SOD})/a(\mathrm{SOD}) + b$ (where *Vs* is the rate of sample containing SOD, *Vc* is the average of at least four controls (SOD = 0); $a = 0.073$, $b = 0.93$).

$\mathrm{SOD} = 0.93\ (Vs/Vc - 1)/1.073 - 0.073\ (Vs/-Vc)$.

The resulting SOD value is multiplied by the dilution factor of the sample and expressed in SOD-525 activity units per ml of extract.

Statistical analysis

Data were analysed using Microsoft Office 2007 (Excel) and Statistical Package for Social Science (SPSS) version 19.0.0 [15], SPSS Inc., Chicago, IL, USA. The following were used to test for significance:

- Student's *t*-test: For comparison of means of two sets of quantitative data;
- Chi-square test: For comparison of two sets of nominal qualitative data;
- One-way analysis of variance (ANOVA): For comparison of more than two sets of quantitative data;
- *Post Hoc*: Tukey Honestly Significant Difference (HSD) test: for multiple intergroup comparisons between more than two sets of qualitative data when ANOVA test shows significant difference;

Probability (p) value was considered to be of statistical significance if it was <0.05.

- Correlation coefficient test: used to determine the degree to which two variables movements are associated.