Original

Protective effects of zinc on oxidative stress enzymes in liver of protein deficient rats

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Abstract

This study was designed to evaluate the protective effects of zinc on the liver activities of antioxidant enzymes in protein-deficient rats. Zinc sulfate at a dose level of 227 mg/l in drinking water was administrated to Sprague Dawley normal control as well as to protein-deficient rats for a total duration of eight weeks. The effects of zinc treatment and protein deficiency alone as well as combined were studied on rat liver antioxidant enzymes which included catalase, glutathione peroxidase (GPX), glutathione reductase (GR), superoxide dismutase (SOD), and glutathione S-transferase (GST). Protein deficiency in normal rats resulted in a significant increase in hepatic lipid peroxidation and in catalase, GPX, GR and GST activity. A significant inhibition in the levels of SOD activity and reduced glutathione (GSH) was observed following protein deficiency in normal rats. Zn treatment to protein deficient animals lowered lipid peroxidation and catalase, GPX and GST activities, and also resulted in a significant elevation in the levels of GSH and SOD activity. The concentration of zinc decreased significantly in protein deficient animals but returned to normal levels when zinc was administered.


Key words: Zinc. Protein deficiency. Liver antioxidants.
edema, fatty liver, atrophy of lymphoid tissues and decreased host immune defense in humans and animals\(^4,5\). Protein deficient diet intake strongly influences the activity of drug metabolizing enzymes\(^6,7\) as well as antioxidant enzymes\(^8,9\). Feeding of a protein-deficient diet to rats has been shown to increase Hpd lipid peroxidation (LPO) and to induce significant changes in the liver activities of catalase, glutathione peroxidase (GPX) and superoxide dismutase (SOD) in the liver\(^10,11\). The significant increase in SOD activity associated with the decrease in plasma ceruloplasmin, antioxidant vitamins and the whole blood GPX activity in protein energy malnourished children suggest that these children are potentially susceptible to high oxidative stress. It has been proposed that free radical-mediated tissue damage may be involved in the pathogenesis of liver diseases, mainly because of the inadequate protective and repair mechanism in protein deficient individuals\(^12\).

Persons afflicted with protein malnutrition are deficient in a variety of micronutrients. Protein deficiency has been shown to decrease the hepatic levels of zinc, manganese, copper, calcium, and magnesium in experimental animals\(^13,14\). Alterations in the levels of trace elements result in number of diseases like hypoalbuminemia, and anemia in malnourished children\(^15\). Bhaskaram and Hemalatha. 1995\(^16\) observed that children suffering from severe protein energy malnutrition have very low levels of the thymulin, hormone which is a sensitive indicator of zinc status in the body, and low leukocyte count, indicating zinc deficiency, which got improved when zinc supplements were provided along with rehabilitation diets. It is known that dietary protein variation affects the absorption and uptake of \(\text{Zn}^{17}\). Zinc supplementation during nutritional rehabilitation of PEM hastens the recovery from protein deficiency and helps in gaining body weight\(^18\). Supplementation of zinc restores serum thymulin activity and improves the nutritional status of elderly people in terms of food intake and serum albumin\(^19,20\).

Zinc has been shown to have an antioxidant potential through the non-enzymatic stabilization of biomembranes and biostructures. Dhawan and Goel, 1994\(^21\) have shown that CCl\(_4\) induced lipid peroxidation in the microsomal fraction of liver homogenates was inhibited by adding zinc to the incubation medium. The present study was undertaken to further elucidate the protective role of zinc on the status of antioxidant enzymes in conditions of protein deficiency.

Materials and methods

Animals

Rats in the weight range of 110-120 g of Sprague Dawley (SD) strain were obtained from the Central Animal Home, Panjab University, Chandigarh. The animals were housed in polypropylene cages in the animal house of the Department of Biophysics, under hygienic conditions and were acclimatized for at least one week before putting them on different treatments. Thereafter, the animals were randomly divided into four groups each having ten animals each.

G-1, Control

Animals in this group served as normal controls and were fed a diet with a normal protein content (18%). Composition of the diet\(^22\) is given in the table below.

G-2, Protein deficient (PD)

Protein deficiency was induced in the animals of this group by feeding a protein-deficient diet with a 8% protein content. Composition of the diet\(^22\) is also given in the table.

G-3 Zinc treated (Zn)

Animals were given zinc in the form of ZnSO\(_4\) at a dose level of 227 mg/L in drinking water and had free access to the normal diet.

G-4, Zn + PD treated

Animals received ZnSO\(_4\) in the drinking water and were given the protein deficient diet.

<table>
<thead>
<tr>
<th>Composition</th>
<th>Normal (18%) Protein Diet</th>
<th>Low (8%) Protein Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein (g)</td>
<td>18</td>
<td>8</td>
</tr>
<tr>
<td>Starch (g)</td>
<td>25</td>
<td>31.5</td>
</tr>
<tr>
<td>Sucrose (g)</td>
<td>25</td>
<td>31.5</td>
</tr>
<tr>
<td>Cellulose (g)</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Corn oil (ml)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Vitamin mixtura (g)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Salt mixture (g)</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

The treatments of rats continued for a period of eight weeks. At the end of the treatment, the animals were weighted and were sacrificed by exsanguination under light anesthesia. Livers were removed immediately and were perfused and rinsed in normal saline (NaCl, 9 g/l/w/v). One lobe was preserved for the determination of various trace elements and the other was processed immediately for various biochemical investigations.

Biochemical determinations

The livers were removed and perfused with normal saline (0.9% WN) to reduce red blood cell contamination. Samples were homogenized in 100 mM potassium phosphate buffer (pH 7.5) containing 0.15 M

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KCl to obtain 25% homogenate, using a motor driven teflon fitted homogenizer. The homogenates were centrifuged in a cold centrifuge (4°C, REMI instruments, Bombay) at 10,000 xg for 30 minutes. The pellets were discarded and the supernatants were again centrifuged at 10,000 xg for 30 minutes. The pellets were discarded and final post mitochondrial supernatant (PMS) was preserved for the estimation of antioxidant enzymes and lipid peroxidation.

The method of Luck, 1971 was used for the estimation of catalase. Glutathione peroxidase was assayed by the method of Flohe and Gurtzler. Glutathione reductase was assayed by the method of Williams and Arscott. The activity of superoxide dismutase was estimated by using the method which is based on the principle of the inhibitory effect of SOD on reduction of nitroblue tetrazolium (NBT) dye by superoxide anions, which are generated by the phomoxidation of hydroxyzylamine hydrochloride (NH₂OH.HCl). Estimation of reduced glutathione was performed in the tissue homogenates of liver by the method of Moron et al. Glutathione-S-Transferase was assayed by the method of Habig et al. Lipid peroxidation was estimated by the method of Ohokawa et al. Protein was measured by the method of Lowry et al.

Zinc concentration

Estimations of zinc concentrations in the liver samples of the different treatment groups were carried using Energy Depressive X-ray Fluorescence (EDXRF), one of the most suitable analytical method to analyze trace elements because of its properties such as non-destructive, sensitivity up to ppm and multielemental analysis.

The liver tissues of all the animals were oven dried at 70°C to a constant weight and then ground with the help of Agate Pestle and Mortar. 300 mg dried powder of the tissue so obtained was weighed and mixed with equivalent mount of Hoechst Wachs (wax) to make self supporting pellets. The pellets were made by using a specially designed pure, steel dye and a hydraulic press from Paul weber, Germany. A force of approximately 45 KN (Kilo newtons) was applied at the dye top in order to make pellets of uniform thickness.

The pellets of tissues were analyzed using an EDXRF X-Lab, 2000 to determine the levels of various elements. The X-lab, 2000 spectrometer involved a 0.4 kw Pd mode Xray tube as source of excitation. The power of the X-ray tube was adjusted on line for each individual measurement by the spectrophotometer software, to secure optimum acquisition parameters for the current analysis.

Presently, different X-ray energies and excitation modes are being used but the most important mode used was of 40 kV and excitation used was polarized X-Ray. A Si (Li) detector coupled with computer (Pentium, 600 MH, software package SPECTRO X-LABpro 2.2) was used to collect the flourescent X-ray spectra from the samples. The X-ray tube, secondary exciter, target and the Si (Li) detector were placed in a triaxial geometry mode. This geometry was used to minimize the background due to scattered photons.

Statistical analysis

The statistical significance of the differences was measured by one way analysis of variance (ANOVA) followed by Newman-Keuls test. The determinations are presented as Mean ± S.D.

Results

The results of all the experiments conducted during the current study we depicted in various tables. All the results of various treatment groups have been compared with their normal controls. Results of zinc + protein deficient (G-4) treated group have been computed with the results of the protein deficient group (G-2).

Body weights

Changes in the body weight of the animals subjected to the different treatments me shown in table I. Body weights of normal control and zinc treated rats, increased progressively throughout the study. Protein deficiency resulted in a significant (p < 0.001) decrea-se in the body weights after eight weeks, when compared to normal control rats. Zinc treatment to the protein deficient rats tended to improve the body weight growth in comparison to protein deficient (G-2) rats but body weights in protein deficient rats were statistically different from normal control rats.

Hepatic protein contents

Table I shows hepatic protein contents in the different experimental groups expressed as mg g⁻¹ tissue. Protein deficient animals, showed a highly significant (P < 0.001) reduction in the hepatic protein contents
as compared to the control group. However, zinc administration to the protein deficient rats helped in raising the hepatic protein contents as compared to their respective controls.

Antioxidant enzymes and lipid peroxidation

The effects of zinc treatment in control and protein deficient rats in different groups on hepatic lipid peroxidation, catalase, glutathione peroxidase, glutathione reductase, superoxide dismutase, reduced glutathione and glutathione-S-transferase are shown in Table II.

Protein deficient rats showed a significant (p < 0.001) increase in hepatic lipid peroxidation, catalase, glutathione peroxidase, glutathione reductase, and glutathione-S-transferase. A significant (p < 0.001) inhibition in the levels of superoxide dismutase activity and reduced glutathione was detected following protein deficiency in normal rats.

Zn treatment to protein deficient animals could lower significantly (p < 0.01) the already raised levels of lipid peroxidation and the activities of enzymes catalase, glutathione peroxidase and glutathione-S-transferase when compared to control animals. Also, Zn treatment to the protein deficient animals resulted in a significant elevation (P < 0.001) in the levels of GSH and SOD activity as compared to their respective controls, thereby indicating its effectiveness in regulating their levels in adverse conditions.

Hepatic concentration of zinc

Table III shows the concentration of zinc in liver tissue of the different groups. Zinc concentration decreased significantly in protein-deficient animals. However zinc levels got elevated to within normal levels in these groups in which zinc was administrated along with other treatments.

Discussion

We observed that the body weights of normal control and zinc treated rats increased progressively throughout the study. Protein deficiency resulted in a significant decline in the body weight after eight weeks, when compared to normal control rats. Loss in body weight is characteristic of protein malnutrition. In an earlier report from our laboratory it has been observed that protein deficiency leads to significant growth retardation in animals. Many other workers have also reported the decrease in body weight due to protein deficiency. It has been observed in these studies that retardation in body weight growth over a period is not due to low food intake but to a deficiency in protein intake. Zinc treatment to the protein-deficient rats tended to improve the body weight growth. Similar protective effects of zinc in improving the body weight gain of the animals have also been reported in other studies, in which radiation or carbon tetrachloride was used to induce liver injury. The protective effects of zinc could be attributed to its ability to reduce collagen accumulation in liver and also it exerts critical physiological role in regulating the structure and function of cells.

Table II

Effect of zinc on lipid peroxidation, reduced glutathione concentration and antioxidant enzyme activities in liver of protein deficient rats

<table>
<thead>
<tr>
<th>Group</th>
<th>LPO (nmol H₂O₂ decomposed min⁻¹ mg⁻¹ protein)</th>
<th>Catalase (nmol NADPH decomposed min⁻¹ mg⁻¹ protein)</th>
<th>GPX (µmol oxidized mg⁻¹ protein)</th>
<th>GR (µmol GSSG reduced hr⁻¹ mg⁻¹ protein)</th>
<th>SOD (IU)</th>
<th>GSH (µmoles of GSH g⁻¹ tissue)</th>
<th>GST (µmol CDNB conjugated min⁻¹ mg⁻¹ protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-1 Control</td>
<td>0.99 ± 0.00</td>
<td>0.63 ± 0.07</td>
<td>0.43 ± 0.04</td>
<td>1.67 ± 0.23</td>
<td>2.24 ± 0.28</td>
<td>2.66 ± 0.08</td>
<td>0.43 ± 0.04</td>
</tr>
<tr>
<td>G-2 Protein-deficient</td>
<td>1.31 ± 0.24⁺</td>
<td>0.76 ± 0.08ᵃ</td>
<td>0.60 ± 0.02ᵇ</td>
<td>2.34 ± 0.14ᵃ</td>
<td>1.73 ± 0.15ᵃ</td>
<td>1.98 ± 0.02ᵇ</td>
<td>0.60 ± 0.02ᵇ</td>
</tr>
<tr>
<td>G-3 Zinc-treated</td>
<td>1.00 ± 0.00</td>
<td>0.68 ± 0.02</td>
<td>0.39 ± 0.02</td>
<td>1.76 ± 0.09</td>
<td>2.27 ± 0.12</td>
<td>2.78 ± 0.21</td>
<td>0.39 ± 0.02</td>
</tr>
<tr>
<td>G-4 Protein-deficient + Zinc</td>
<td>0.99 ± 0.01⁺</td>
<td>0.64 ± 0.06ᵃ</td>
<td>0.42 ± 0.03ᵇ</td>
<td>2.03 ± 0.29ᵃ</td>
<td>2.10 ± 0.04ᵃ</td>
<td>2.60 ± 0.09ᵇ</td>
<td>0.42 ± 0.03ᵇ</td>
</tr>
</tbody>
</table>

Table III

Levels of zinc following zinc treatment in protein-deficient rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Zinc (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-1 Control</td>
<td>58 ± 6</td>
</tr>
<tr>
<td>G-2 Protein-deficient</td>
<td>40 ± 5ᵃ</td>
</tr>
<tr>
<td>G-3 Zinc-treated</td>
<td>58 ± 8</td>
</tr>
<tr>
<td>G-4 Protein-deficient + Zinc</td>
<td>51 ± 5ᵇ</td>
</tr>
</tbody>
</table>

Values me Mean ± SD.
⁺p < 0.05, ⁺p < 0.01 and ⁿp < 0.001 in comparison to G-1.
ᵖ < 0.05, ᵃp < 0.01 and ᵄp < 0.001 comparison of G-4 with G-2.
In our study, the levels of zinc decreased in protein-deficient rats, but returned to normal following zinc supplementation. The observation of depressed Zn levels in the liver of protein deficient rats in the present investigation we in conformity with previous studies. Abnormalities in zinc metabolism leading to its deficiency are generally attributed to various factors like, malabsorption, malnutrition, decreased intestinal zinc binding factors or the increased excretion of the zinc via the gastrointestinal tract or via urine are of common occurrence in chronic liver disorders. The results of the current study for lowered zinc concentrations could be explained on the basis that either it is excessively being utilized in providing antioxidant defense mechanism or there is some defect in the absorption/metabolism of zinc in toxic conditions created by protein deficiency. Studies carried out in animals and humans had shown that zinc is essential for utilization of amino acids. Conversely, protein malnutrition plays a major role on liver zinc depletion. Zinc has been found to be associated with metal binding proteins that are known to regulate the functions of zinc as well as copper. Metallothionein also plays a role in the detoxification of heavy metals and stabilize membranes.

Protein-deficient treatment groups, showed a highly significant reduction in the protein content as compared to normal control group which is in agreement with the earlier reports. Davenport et al, 1994 demonstrated that in protein deficient states, the reduction in serum albumin contents were due to depletion in amino acid precursors of albumin synthesis. However, zinc administration to the protein deficient rats helped in raising the hepatic protein contents (p < 0.001) and were brought to normal limits as compared to their respective controls. This property of Zn could be attributed to its role in the induction of metallothionein (Zn binding protein) thereby regulating the amino acid precursors for albumin synthesis.

Lipid peroxidation is the process of oxidative degradation of polyunsaturated fatty acids (PUFA). Its occurrence in biological membranes causes impaired membrane function, impaired structural integrity, decrease in fluidity, and inactivation of a number of membrane bound enzymes and protein receptors. Lipid peroxidation is an autocatalytic free-radical process and could be responsible for DNA damage.

A significant increase in malondialdehyde products was observed in the protein deficient groups in the present study which suggested that low protein diet intake might result in enhanced lipid peroxidation in liver. These results seems to be in agreement with previous findings suggesting that the rats fed on a low protein diet might be more susceptible to peroxidative tissue damage under the influence of oxidative stress. The increased lipo peroxidation could be attributed to the reduction in detoxifying hyperperoxides in protein deficient conditions. Moreover, the degree of depressions of detoxifying hyperperoxides in protein deficient conditions might also be correlated with the degree of protein deficiency.

The normalization of lipo peroxidation due to Zn administration could be attributed to its antiperoxidative property. Studies have shown that Zn causes inhibition of both endogenous as well as induced lipid peroxidation to stabilize biomembranes. Further, the levels of zinc which were reduced in low protein conditions got maintained by its supplementation and this apparently contributed to a reduction in lipid peroxidation.

To protect themselves against free radicals, cells have developed antioxidant defenses and repair systems which prevent the accumulation of oxidatively damaged molecules. The antioxidant defense system include enzymes like glutathione peroxidase (GPx), catalase, glutathione reductase (GR), glutathione-s-transferase (GST), superoxide dismutase (SOD), as well as small molecules such as ascorbic acid, reduced glutathione (GSH) and uric acid. Catalase is a ubiquitous enzyme and is a major component in primary antioxidant enzyme system, which catalyzes the decomposition of H₂O₂ to H₂O and sharing this function with glutathione peroxidase (GPx). Glutathione peroxidase on the other hand is located in the cytosol and mitochondrial matrix and catalyzes the reduction of H₂O₂ and lipid and nonlipid hydroperoxides to oxidized glutathione (GSSG) using two molecules of GSH. Further oxidized, GSSG is reduced back to GSH by glutathione reductase (GR), which utilizes NADPH regenerated by glucose-6-phosphate dehydrogenase.

In the present study, after subjecting the rats to protein deficiency the hepatic activity of catalase, glutathione peroxidase and glutathione reductase got raised but superoxide dismutase activity was found to be inhibited. Darmon et al, 1993 also observed an increase in catalase activity in low protein diet fed rats. Zhu et al, 1993 accounted the high levels of GPx following protein deficiency due to its low utilization and increase in synthesis. An enhanced level of glutathione reductase has been reported earlier in rats fed protein restricted diet. The observed elevation in the activities of both GPx and GR in the present study under low protein diet may be due to enhanced synthesis of these enzymes, which are actively involved in reducing the H₂O₂ generation.

The observed increase in lipid peroxidation in the protein deficient group seems to be associated with a decrease in SOD activity, as SOD inhibits hydrogen peroxide by scavenging free oxide molecule. Our results regarding the significant decrease of SOD following restriction of protein diet are in agreement with earlier reports. Following zinc treatment the altered levels of enzymes tended to be normalized because of the antioxidant property of zinc.

Glutathione-s-transferases (GSTs) form a group of enzymes that are present in high concentrations in the cytosol and catalyze a wide variety of substitution reactions in which glutathione (GSH) replaces an easily dis-
placed group on the xenobiotic, and thus prevents the subsequent toxic reactions\(^5\). This reaction involves a compound with an electrophilic atom and GST facilitates the nucleophilic attack of glutathione thiolate on this electron deficient atom of the hydrophobic compound. GSH plays an important role in intracellular protection against toxic compounds, reactive oxygen species, and free radicals\(^6\). Reduced glutathione (GSH) protects the liver microsomes against the effects of reactive (peroxides and oxygen) intermediates which are formed by Cytochrome P450 system as well as lipid peroxidation\(^7\).

In the present study the low protein diet caused a marked decrease in the levels of GSH which is in agreement with earlier studies\(^8,9\). Ayla et al\(^9\) observed that in rats fed on a low protein diet, supplemented with all essential amino acids except methionine, there was a decrease of GSH levels. They proposed that low intracellular concentration of cysteine available for GSH synthesis and feed back inhibition of gamma glutamyl cysteine synthetase may be responsible for inhibition in the activities under protein deficient conditions. The low protein diet results in increase of hepatic levels of glutathione degrading enzyme, gamma glutamyl transferase (\(\gamma\)-GT) and, thus, a decreased concentration of glutathione\(^9\). Because GSH is an important component of the detoxification mechanism, its lowered concentration in protein-deficient conditions would, therefore, lead to decreased detoxification capacity of liver. Further reduction in GSH levels in protein deficient is understandable in the light of elevation of GPx under these conditions. The present observations of a decline in GSH levels in protein deficient groups are in coherence with earlier reports\(^9\) and can be attributed to the activation of \(\gamma\)-GT to replenish intracellular glutathione on the sinusoidal surface of the liver cells\(^9\).

We have also observed an increase in GST activity following protein deficiency treatment. Ramdath and Golden\(^8\) reported a similar increase in malnourished children. Cho et al\(^9\) stated that the antioxidant response element (ARE)-binding activity of protein-calorie malnutrition rats gets increased, which in turn results in activation of certain GST mRNAs and a higher GST activity.

The observed normalization of GSH levels and GST activity following Zn treatment could be because of its property to induce metallothionein (S-rich protein) as a free radical scavenger, or its indirect action in reducing the levels of oxygen reactive species\(^9\), however the precise mechanism for these actions remains to be elucidated.

In conclusion, the present study highlights the protective role of zinc in maintaining the activities of enzymes involved in oxidative stress induced in conditions of protein deficiency.

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