Effect and possible role of Zn treatment in LEC rats, an animal model of Wilson’s disease

Alessandro Santona, Paola Irato, Valentina Medici, Renata D’Incà, Vincenzo Albergoni, Giacomo Carlo Sturniolo

*Department of Biology, University of Padova, via U. Bassi, 58/B, 35131 Padua, Italy
bDepartment of Surgical and Gastroenterological Sciences, University of Padova, Padua, Italy

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Abstract

The effect of oral zinc (Zn) treatment was studied in the liver, kidneys and intestine of Long–Evans Cinnamon (LEC) rats in relation to metals interaction and concentration of metallothionein (MT) and glutathione (GSH). We also investigated the change in the activity of antioxidant enzymes and determined the biochemical profile in the blood and metal levels in urine. We showed that the Zn-treated group had higher levels of MT in the hepatic and intestinal cells compared to both untreated and basal groups. Tissue Zn concentrations were significantly higher in the Zn-treated group compared to those untreated and basal, whereas Cu and Fe concentrations decreased. The antioxidant enzyme activities in the Zn-treated group did not change significantly with respect to those in the basal group, except for hepatic glutathione peroxidase activity. Moreover, the biochemical data in the blood of Zn-treated group clearly ascertain no liver damage. These observations suggest an important role for Zn in relation not only to its ability to compete with other metals at the level of absorption in the gastrointestinal tract producing a decrease in the hepatic and renal Cu and Fe deposits, but also to MT induction as free radical scavenger.

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1. Introduction

The Long–Evans Cinnamon (LEC) is an inbred strain of Long–Evans rat which spontaneously develops acute hepatitis at around 3–4 months after birth and then there is a sudden onset of spontaneous hepatitis in LEC rats. Both sexes are affected, but females are more vulnerable to fulminant hepatitis. Kasai et al. [1] reported that 60–70% of the males survive fulminant hepatitis, whereas only 20% of the females do. These animals have a mutation in the gene homologous to the human Wilson’s disease (WD) gene, *ATP7B* [2]. A defect in the final product (Cu-binding P-ATPase) of the gene, ATP7B, may cause a defect of Cu excretion from hepatocyte into bile, resulting in hepatic Cu accumulation [3]. Besides elevated hepatic Cu accumulation similar to that occurring in WD, LEC rats also show abnormal hepatic iron (Fe) overload depending on age [4]. Fe and Cu can induce free radical production, DNA damage, protein or peptide modification, oxidation of low-density lipoproteins and catalyze peroxidation of membrane lipids [5,6]. Yamamoto et al. [7] observed that increased levels of redox active free Cu in the liver of LEC rats catalyze Fenton-type reactions, thus producing hydroxyl radicals and lipid peroxidation (LPO) and causing the reduction of selenium-dependent glutathione peroxidase (Se-GPX) activity and the inactivation of catalase (CAT). Significantly decreased Se-GPX, CAT and reduced glutathione (GSH) and increased superoxide dismutase (SOD) activity in the liver of acute Cu-poisoned rats have also been reported [8]. On the other hand, Freedman et al. [9], using a wild-type hepatoma cell line (HAC) selected for Cu resistance, reported that over 60% of the cytosolic Cu accumulated by these cells was bound to GSH and that resistance was well correlated with elevated levels of the
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tripeptide. Some studies have reported on the ability of zinc (Zn) to interact with Cu and Fe decreasing metal content in tissues and retarding oxidative processes [10,11]. Antagonism of redox-active transition metals, such as Cu and Fe, and site-specific reactions has led to the theory that Zn may be capable of reducing cellular injury that might have a component of site-specific oxidative damage [12].

The effectiveness of Zn in controlling Cu-inducing liver damage has been documented in WD patients [13]. The action mechanisms of Zn are not fully elucidated but metallothionein (MT) induction in the gastrointestinal mucosal cells seems to be responsible for trapping Cu in the enterocytes. Thus, Zn acts by forming a mucosal block for Cu absorption and increases the fecal excretion of Cu [14]. Moreover, Zn-induced MT has been proposed to play a role in the cellular protection against damage caused by excessive Cu accumulated in tissues [15].

To verify the effectiveness of Zn therapy, we compared the untreated with the Zn-treated LEC rat group (95 days old) determining the effect of 60 days treatment on MT, GSH, and trace element (Zn, Cu and Fe) content in the liver, kidneys and intestine. We also studied the changes in the activity of hepatic and renal free radical-metabolizing enzymes, Cu,Zn-SOD, Se-GPX, total glutathione peroxidase (T-GPX) and CAT and determined the biochemical profile and metal levels in blood and urine, respectively. As for the initial conditions, we measured the same parameters in the basal group (35 days old).

2. Materials and methods

2.1. Animals

Thirty male LEC rats (35 days old, 90 g body weight) from Charles River Japan (Tokyo, Japan) were used for the study. They were kept on a standard laboratory diet (Morini MIL GLP diets) containing 11.7 mg Cu/Kg and 67.5 mg Zn/Kg and deionized water. They were housed in comfortable cages at 20 °C with a 12-h light–dark cycle. Zn acetate supplement was dissolved in 2% glucose solution in distilled water.

We divided the 30 rats as follows: 13 rats received by gavage an oral dose of Zn acetate 50 mg/ml daily for 60 days (Zn-treated group); 13 rats received daily by gavage an oral dose of glucose solution 0.02 mg/ml daily for 60 days (untreated group); 4 rats received no treatment and they were sacrificed 35 days after birth (basal group).

Rats were sacrificed under diethyl ether, liver, kidneys and intestine were quickly removed, washed in saline solution repeatedly, frozen with liquid nitrogen and stored at −80 °C until used.

All procedures were carried out on laboratory rats according to the regulations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Determination of trace elements, MT and GSH contents in hepatic, renal and intestinal tissues

A part of the tissues was homogenized in 4 vol of 20 mM Tris–HCl buffer, pH 8.6, supplemented with 0.006 mM leupeptine, 0.5 mM phenylmethylsulphonylfluoride (PMSF) as antiproteolitic agents, and 0.01% β-mercaptoethanol as reducing agent. The homogenate was centrifuged at 4 °C at 50 400 × g for 50 min, and the resulting supernatant was used for Cu, Fe and Zn estimation by atomic absorption spectrophotometry (Perkin-Elmer mod. 4000) and MT quantification using the silver saturation method [16]. The trace elements and MT amount in the supernatant was expressed relative to total soluble cell protein assayed by the Lowry method [17]. The metals were also determined in the homogenate and expressed relative to dry weight.

For the GSH assays, tissue samples were homogenized in 5 vol of 5% 5-sulfosalicylic acid. To obtain supernatant solutions, the homogenates were centrifuged at 16100 × g for 15 min at 4 °C. GSH was determined according to the method of Anderson [18]. Values are reported in nanomoles GSH per gram fresh weight.

2.3. Determination of trace elements in urine

To eliminate organic matter, 1 ml aliquots of urine sample were digested in Teflon vessels with 0.5 ml nitric acid AristaR in a microwave CEM mod. MDS-2000. Cu, Fe and Zn were determined utilizing synthetic urine [19], used as standard solution, and assayed by atomic absorption spectrophotometry (Perkin-Elmer mod. 4000).

2.4. Determination of antioxidant enzymes activities

For the enzyme assays, tissue samples were homogenized in 4 vol of Tris–HCl 10 mM (pH 7.6), DTT 1 mM, KCl 0.15 M, sucrose 0.5 M. The homogenates were centrifuged at 52 000 × g for 50 min at 4 °C. Spectrophotometric measurements were carried out on a Perkin-Elmer Lambda 10 spectrophotometer. Assays were performed in duplicate or triplicate and assay temperatures were 20 °C.

The assay for the SOD activity utilizes the reduction of nitro blue tetrazolium (NBT) as detector of O2− and defines SOD activity in terms of its ability to inhibit the reduction of NBT due to O2−. A unit was defined as the amount needed to cause half-maximal inhibition that can be achieved by SOD [20]. CAT was determined according to the method of Aebi [21] after the decomposition of H2O2 at 240 nm. One unit is defined as the amount of enzyme that catalyzed the dismutation of 1 μmol of H2O2 per minute.

The measurement of the Se-GPX and T-GPX activities in supernatant was determined by the method of Livingstone et al. [22] using H2O2 or cumene hydroperoxide (CHP) as substrate, respectively. One unit of GPX equals 1 μmol glutathione oxidised per minute.

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All these values were referred to total proteins determined by the folin–phenol reagent [17].

2.5. Analyses of biochemical profiles in serum

Activities of aspartate amino transferase (AST) and alanine amino transferase (ALT) were determined at 340 nm in units per liter of serum using an automatic analyzer (Hitachi mod. 747), whereas total bilirubin (T-Bil) concentrations were measured at 570 nm in micromoles per liter of serum. All procedures were carried out using a clinical guide to laboratory tests [23,24]. Moreover in the organs, Cu concentration in the Zn-treated group was similar to those in the basal one, whereas 

2.6. Statistical analysis

All measurements were made in duplicate and results are reported as means ± S.D. Statistical analysis was performed with the Primer statistical program. Statistical differences were calculated with one-way analysis of variance (ANOVA) with a Student–Newman–Keuls follow-up test. Significant difference was accepted at $P < 0.05$.

3. Results

3.1. Concentrations of trace elements in the organs and urine

The trace element concentrations in hepatic, renal and intestinal supernatant and urine of Zn-treated, untreated and basal groups are shown in Tables 1 and 2.

Zn concentrations were significantly higher in the Zn-treated group than in both untreated and basal groups in all organs considered. In particular, we observed that, in the hepatic, renal and intestinal supernatant fraction, Zn levels were 1.25-, 2- and 2-fold, respectively, higher than both the untreated and basal groups. Instead, Cu and Fe levels were significantly lower in the Zn-treated group than in the untreated group concentrations and 3- and 3.3-fold higher than in the untreated group concentrations and 3- and 3.3-fold, respectively, than the basal one. The renal MT concentrations in the Zn-treated group were also 3.3-fold higher than in the basal group, whereas there were no significant differences compared to those in the untreated group. On the other hand, the hepatic and renal GSH concentrations in the Zn-treated group were significantly lower (0.55- and 0.67-fold, respectively) than in the untreated one. In the kidneys, GSH concentrations in the

3.2. Quantification of MT and GSH in the organs

As shown in Table 1, the hepatic and intestinal MT concentrations in the Zn-treated group were 1.3-fold

<table>
<thead>
<tr>
<th>Liver</th>
<th>Cu</th>
<th>Zn</th>
<th>Fe</th>
<th>MT</th>
<th>GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal group (Bg)</td>
<td>1.3 ± 0.2</td>
<td>0.4 ± 0.0</td>
<td>0.8 ± 0.2</td>
<td>4.2 ± 0.9</td>
<td>3810.5 ± 568.6</td>
</tr>
<tr>
<td>Untreated group (Ug)</td>
<td>2.5 ± 0.6</td>
<td>0.4 ± 0.2</td>
<td>2.1 ± 1.0</td>
<td>9.6 ± 2.2</td>
<td>5987.2 ± 1771.8</td>
</tr>
<tr>
<td>Treated group (Tg)</td>
<td>1.6 ± 0.3</td>
<td>0.5 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>12.3 ± 3.3</td>
<td>3390.6 ± 1002.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Kidneys</th>
<th>Cu</th>
<th>Zn</th>
<th>Fe</th>
<th>MT</th>
<th>GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal group (Bg)</td>
<td>0.04 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>0.3 ± 0.1</td>
<td>1.7 ± 0.3</td>
<td>2970.7 ± 10.8</td>
</tr>
<tr>
<td>Untreated group (Ug)</td>
<td>1.8 ± 0.5</td>
<td>0.2 ± 0.0</td>
<td>0.5 ± 0.1</td>
<td>6.5 ± 1.5</td>
<td>7860.6 ± 14.8</td>
</tr>
<tr>
<td>Treated group (Tg)</td>
<td>0.07 ± 0.0</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.0</td>
<td>5.6 ± 0.7</td>
<td>5310.2 ± 27.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Intestine</th>
<th>Cu</th>
<th>Zn</th>
<th>Fe</th>
<th>MT</th>
<th>GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal group (Bg)</td>
<td>0.05 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>2.7 ± 0.8</td>
<td>1550.0 ± 286.5</td>
</tr>
<tr>
<td>Untreated group (Ug)</td>
<td>0.05 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>7.1 ± 2.0</td>
<td>1124.6 ± 417.6</td>
</tr>
<tr>
<td>Treated group (Tg)</td>
<td>0.03 ± 0.0</td>
<td>0.4 ± 0.1</td>
<td>0.1 ± 0.0</td>
<td>8.9 ± 1.8</td>
<td>1114.7 ± 670.9</td>
</tr>
</tbody>
</table>

Values are indicated as mean ± S.D. in micrograms per milligram protein for metals and MTs and nanomoles per milligram fresh weight for GSH. Significantly different for Bg = Basal group; Ug = Untreated group; Tg = Treated group.

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Zn-treated group were significantly higher (1.7-fold) than in the basal group. Moreover, there were slight, but not significant differences in the intestinal GSH concentrations among the three groups. Furthermore, both hepatic and renal MT and GSH concentrations in the untreated group were significantly higher compared to those in the basal one. In particular, hepatic and renal MT concentrations were 2.3- and 3.8-fold, respectively, higher compared to those in the basal group and GSH concentrations were 1.6- and 2.6-fold, respectively, than those in the basal group.

3.3. Antioxidant enzymes and serum biochemical profile

Fig. 1 shows that Cu,Zn-SOD activity was similar in the liver of the three groups, whereas renal enzyme activity levels were significantly different between Zn-treated and untreated groups. In particular, renal Cu,Zn-SOD activity was significantly higher (1.4-fold) in the untreated group compared to the Zn-treated group. On the other hand, CAT activity in the liver of Zn-treated group was 3.1-fold higher than in untreated one, whereas no significant difference was noted between those Zn-treated and basal. Moreover, CAT activity in the untreated group was significantly lower (0.3-fold) compared to that in the basal group. In the kidneys, CAT activity was at a similar level among the three groups.

In the liver, the levels of Se-GPX activity were significantly lower (0.6-fold) in the Zn-treated group than in the basal ones, whereas no significant difference was observed between the same groups in the kidneys. Even, no significant difference was observed between Zn-treated and untreated groups in the liver, whereas in the kidneys, Se-GPX activity in the untreated group was 1.7-fold higher with respect to that in the Zn-treated group. With regard to T-GPX activity, we observed in the liver, enzyme activity levels were significantly higher (1.73- and 1.44-fold, respectively) in the untreated group than both the Zn-treated and basal groups, whereas no significant difference was observed between Zn-treated and basal groups. In the kidneys, T-GPX activity was significantly higher (2.9- and

2.7-fold, respectively) in the untreated group than both the Zn-treated and basal groups. Serum AST and ALT activities, and T-Bil concentrations in the Zn-treated rats were almost the same as those in the
basal rats. Untreated rats showed a significant increase in AST (523.8 ± 147.8) and ALT (336.2 ± 138.4) activities, and T-Bil concentrations (621.5 ± 171.1), as summarized in Table 3.

4. Discussion

In our report, we describe the effect of 60 days treatment with Zn acetate on MT, GSH and metal content (Zn, Cu and Fe), evaluate the changes in the activity of antioxidant enzymes and determine the biochemical serum profile and trace elements concentrations in urine of LEC rats, an animal model of WD.

Several papers have discussed the relationship between oxidative stress and hepatic accumulation of toxic metals in LEC rats [26–28]. However, none of the papers investigated the antioxidant effect and possible role of Zn supplementation in these rats. The role of Zn in protecting biological structures from free radical damage may be due to several factors: maintaining an adequate level of MTs, which are also free radical scavengers; as an essential component of Cu, Zn-SOD; as a protective agent for thiols and other chemical groups [29].

Our results show that, in general, treatment with zinc acetate increases tissue Zn and MT contents and decreases Cu and Fe concentrations in the liver, kidney and intestine supernatant fraction. The reduction of Cu and Fe is accomplished through mechanism changes by decreasing the intestinal absorption of Cu and Fe and by enhancing metal excretion [30]. Cousins and McMahon [31] reported that the Fe transporter Nramp 2 (divalent cation transporter -1) also exhibits Zn transport capabilities. We suppose that in the presence of large amounts of Zn, this transporter might bind more Zn than Fe causing a decrease in Fe content in tissues. It is well known that Zn is thought to be a more potent inducer of MT than Cu; however, MT has much higher affinity for Cu than Zn, resulting in Cu sequestration within enterocytes [32]. The complexation of Cu prevents its serosal transfer resulting in a decrease in serum Cu levels and its excretion into the feces along with intestinal cells [33]. In urine, Cu and Fe concentrations decreased significantly compared to untreated one, whereas Cu concentrations were significantly higher in the Zn-treated group than in basal groups. We postulated that Zn, at high doses, might interact with Cu for the ligand sites on MT, as suggested by our previous reports [34], decreasing metal content in both hepatic and renal cells. Moreover, Zn-induced MT protects against accumulated Cu toxicity in the liver and kidneys, suggesting that this protein might be important in the cellular defence against oxidative stress.

Whereas MT induction plays a part in Zn protective action, other factors, such as GSH, may also be involved because protection is offered even in MT-null mice [35]. Our results show that the hepatic GSH concentrations were at similar levels in both the Zn-treated and basal groups. Maintenance of normal GSH status is essential for Zn protection, as inhibition of GSH synthesis abolishes this protection. This proposal is based on observations that GSH depletion potentiates metal toxicity in rats, mice, and cultured cells [36]. In our work, we observed that the hepatic and renal GSH concentrations in the untreated group, in which there was an abnormal Cu accumulation, were 1.8- and 1.5-fold, respectively, higher than the Zn-treated group and 1.6- and 2.6-fold, respectively, higher than the basal groups. Jiménez and Speisky [37], in an in vitro study on the effects of Cu ions on the free radical-scavenging properties of GSH, showed that besides preventing the occurrence of a redox-active Cu, a Cu–GSH complex could per se also act to scavenge free-radical species. GSH sequesters redox active Cu ions preventing them from catalyzing free radical generation. The formation of Cu–GSH complexes could be regarded as an additional mechanism in the protection of cells against excessive Cu exposure.

GSH is also a substrate for GSH peroxidases (Se-GPX and T-GPX), enzymes capable of both removing hydrogen peroxide from the cells and repairing peroxidatively damaged membranes. Downey et al. [38] showed that the LEC rat possesses a deficiency in hepatic selenium (Se) and reduced levels of both hepatic Se-GPX RNA, and Se-GPX activity. Around 65% of dietary Se in the rat is thought to be associated with the liver, and the cause of hepatic Se deficiency in LEC rats is elusive. Our data demonstrate that the levels of hepatic Se-GPX activity were significantly lower in the Zn-treated group than in the basal one, suggesting that this deficiency may be caused by the increased Se excretion and/or decreased Se absorption. It is also possible that Zn and Se can compete with each other for binding to a number of proteins leading to a deficiency in cellular Se. It is also true, that Se-GPX activity was significantly lower in the untreated group than in the basal one. Other studies are needed to clarify the role of Zn and Cu in relation to the changes of Se-GPX activity in LEC rats. We also observed that the levels of both Se-GPX and CAT activity in the liver were significantly higher (1.3- and 3.6-fold, respectively) in basal group than in untreated animals. Yamamoto et al. [7] reported that in the liver of LEC rats with excess amounts of Cu, the increased LPO and the ROSs generated from free Cu are thought to be involved in the inactivation of CAT in much the same way as in Indian childhood cirrhosis, a disease which shares similar types with WD. Se-GPX deficiency in the LEC rat is likely to exacerbate the anaemia that occurs concomitantly with acute hepatitis [39]. On the other hand, T-GPX activity levels were significantly higher in the untreated group than both the Zn-treated and basal groups in the liver and kidneys, whereas no significant difference was observed between Zn-treated and basal groups. It is known that hydrogen peroxide (H$_2$O$_2$) and consequently hydroxyl radical are generated during Cu-catalyzed GSH oxidation. The cyclic regeneration of GSH from GSSG by NADPH-dependent GSSG-R may cause sustained generation of
hydroxyl radical in the presence of excess free Cu. An increased activity of total T-GPX in the untreated group may be explained as a protective cellular mechanism against an increased generation of H₂O₂ caused by both Cu and Fe accumulation in tissues.

No significant change was noted in Cu,Zn-SOD activity in the liver and kidneys among the three groups, except for renal enzyme activity levels between Zn-treated and untreated groups. Similar findings were found by Ohhira et al. [26] in the liver of LEC rats before and after the onset of hepatitis. The significant increase of Cu,Zn-SOD activity observed in the kidneys of the untreated group probably indicates a response to the excessive production of superoxide anion (O₂⁻).

Moreover, we observed an abnormal serum T-Bil concentration in the untreated group, whereas there were no significant differences between Zn-treated and basal groups. Serum T-Bil concentration showed a similar pattern to serum ALT and AST. These findings accorded well with those of Nomiyama et al. [40] who noted a significant increase in serum activities in the period between 12–16 weeks and upon the onset of jaundice in LEC rats.

The maintenance on basal levels of the antioxidant enzymes activities, of GSH content and other serum biochemical parameters in Zn-treated group strongly support the hypothesis of ability of Zn to retard oxidative mechanisms, suggesting a role in modulating ROSs. Zn treatment interrupts the pathological process that leads to induction of the antioxidants in the diseased tissue. This effect of Zn is based on the observation that during the course of the experiment, over 50% of untreated rats get the jaundice acute hepatitis and then die, whereas all Zn-treated rats survived. We believe that this protective effect of Zn is related not only to the ability of Zn to compete with other metals at the level of absorption in the gastrointestinal tract producing a decrease in the hepatic and renal Cu and Fe deposits, but also to MT induction as free radical scavenger or both hypotheses could be true. In this contest, LEC rats seem to be a good animal model which elucidates the pathological relationship among free radicals, metal toxicity, and hepatorenal injury. We postulate that these findings are useful in improving our knowledge on pathophysiology of WD.

Further research is being carried out to evaluate the antioxidant properties of Zn and Zn-MT in relation to apoptotic processes as cellular response to DNA damage by free radicals.

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References

enzymes in the digestive gland of the common mussel *Mytilus edulis*,


