Mineral metabolism in dimethylnitrosamine-induced hepatic fibrosis

Joseph George *

Department of Biochemistry, Central Leather Research Institute, Adyar, Madras 600 020, India

Received 23 March 2006; received in revised form 30 June 2006; accepted 15 July 2006

Available online 26 July 2006

Abstract

Objectives: Complications such as ascites during the pathogenesis of hepatic fibrosis and cirrhosis may lead to several abnormalities in mineral metabolism. In the present investigation, we have monitored serum and liver concentrations of calcium, magnesium, sodium and potassium during experimentally induced hepatic fibrosis in rats.

Design and methods: The liver injury was induced by intraperitoneal injections of dimethylnitrosamine (DMN; N-nitrosodimethylamine, NDMA) in doses 1 mg/100 g body weight on 3 consecutive days of each week over a period of 21 days. Calcium, magnesium, sodium and potassium were measured by atomic absorption spectrophotometry in the serum and liver on days 7, 14 and 21 after the start of DMN administration.

Results: Negative correlations were observed between liver function tests and serum mineral levels, except with albumin. Calcium, magnesium, potassium and sodium concentrations in the serum were decreased after the induction of liver injury. The liver calcium content was increased after DMN treatment. No change occurred in liver sodium content. However, magnesium and potassium content was significantly reduced in the hepatic tissue.

Conclusions: The results suggest that DMN-induced hepatic fibrosis plays certain role in the alteration of essential elements. The low levels of albumin and the related ascites may be one of the major causes of the imbalance of mineral metabolism in hepatic fibrosis and further aggravation of the disease.

Keywords: Dimethylnitrosamine; N-nitrosodimethylamine; Mineral metabolism; Hepatic fibrosis; Liver cirrhosis; Essential elements; Minerals; Ascites

Introduction

Several mineral metabolism disorders have been described in association with hepatic diseases, but their cause, significance and relationship to clinical complications have yet to be identified. Many elements play important roles in the living body as components of metalloproteins and metalloenzymes as well as enzyme cofactors [1]. Since the metabolism of these compounds takes place mainly in the liver, studies of alterations of minerals and trace elements during liver disorders have been of considerable importance in recent years. However, the factors associated with liver diseases and mineral metabolism are still obscure. Since hepatic fibrosis and cirrhosis lead to functional impairment of liver tissue, alterations in the levels of important minerals may contribute to the pathogenesis of hepatic fibrosis.

Sodium has a major role in the development of ascites in patients with liver cirrhosis. Impaired water and sodium excretion has been incriminated in the pathogenesis of ascites formation [2–4]. Hyponatremia is a common phenomenon in patients with hepatic cirrhosis [5–7]. Potassium is the principal intracellular cation and its metabolism may be altered during liver fibrosis. The frequent finding of hypokalemia in liver diseases [8] is often attributed to total body potassium deficiency.

Disturbances in calcium metabolism have been reported in hepatic fibrosis and related diseases [9]. Magnesium is required for the synthesis of all proteins and nucleic acids and also

* Present address: Division of Molecular Medicine, Department of Medicine, Columbia University, 630 West 168th Street, New York, NY 10032, USA. Fax: +1 212 305 1188.

E-mail address: jgeorge40@hotmail.com.
involved in carbohydrate metabolism. Data are scanty regarding the involvement of magnesium in liver diseases.

Even though considerable data are available with regard to the role of minerals in liver diseases, the correlation between alteration of minerals and development of hepatic fibrosis is not clear. Furthermore, very little information is available concerning changes in minerals in the liver during hepatic fibrosis and other liver disorders. It was reported that dimethylnitrosamine-induced liver injury in rats is a suitable and reproducible animal model for studying various events associated with development of hepatic fibrosis and alcoholic cirrhosis in human beings [10,11]. This model is also shown to produce many decompensating features of human hepatic fibrosis such as portal hypertension, ascites, hypoproteinemia and biochemical abnormalities [12,13]. Furthermore, it was reported that DMN-induced liver injury in rats appears to be a potential animal model for early human cirrhosis and may serve as a convenient procedure for screening antifibrotic agents [14]. Therefore, concentrations of biochemically and physiologically important minerals such as calcium, magnesium, potassium and sodium were studied in serum and liver samples during the pathogenesis of DMN-induced hepatic fibrosis in adult male albino rats and the data correlated with liver functions.

Materials and methods

Chemicals

Dimethylnitrosamine (N-nitrosodimethylamine) and lanthanum oxide were purchased from Sigma Chemical Company, St. Louis, MO, USA. Specpure sodium chloride, potassium chloride, calcium carbonate and magnesium metal were procured from Johnson Matthey Chemicals, Orchard Road, Royston, Hertfordshire, England. Ethylene glycol monomethyl ether (methyl cellosolve) was procured from Fluka (Switzerland), and 2-p-dimethyaminobenzaldehyde was from E. Merck (Darmstadt, Germany). All other chemicals and reagents used were of either spectroscopical or analytical grade.

Animals

The animal protocol was approved by the institutional animal care and use committee for the maintenance and use of laboratory animals. Adult male albino rats of the Wistar strain, aged about 3 months and weighing between 180 and 200 g were used for the experiment. The animals were bred and maintained under 12-h light/12-h dark cycles in an air-conditioned animal house, with commercial rat feed pellets (Hindustan Lever, Bombay, India) and water available ad libitum. They were kept in polypropylene cages with a wire mesh top and a hygienic bed of husk.

Induction of hepatic fibrosis

Hepatic fibrosis was induced by intraperitoneal injections of dimethylnitrosamine in doses of 1 mg (10 μL diluted to 1 mL with 0.15 mol/L sterile NaCl)/100 g body weight. The injections were given on three consecutive days of each week over a period of 21 days. Control animals also received an equal volume of 0.15 M NaCl without DMN. The injections were given without anesthesia. The body weight of all the experimental animals was monitored throughout the study. Treated animals were sacrificed on days 7, 14 and 21 from the beginning of exposure by decapitation. A few of the control animals were sacrificed at the beginning of the experiment and the remaining together with the treated animals on days 7, 14 and 21 and the pooled mean value was used as control. The control and the 7th day group comprised 16 rats each, while the 14th and 21st day group comprised 12 and 10 rats, respectively. All the animals were anesthetized before sacrifice using diethyl ether in an air controlled chamber. Blood was collected from the orbital sinus of the animal by piercing a heparinized capillary tube under anesthesia. Blood was also collected from a deep cut made on the right jugular vein on the neck. The blood was allowed to clot at 37°C for 1 h and serum was separated by centrifugation at 2000×g for 10 min. A portion of the serum sample was used immediately for clinical laboratory tests and the remaining was stored in screw capped polypropylene vials at −20°C. The liver tissue was quickly removed, weighed and a median lobe of 3 mm thick was instantly fixed in 10% phosphate-buffered formalin for histopathological studies. Another portion of the liver was frozen at −80°C for biochemical studies. The remaining liver tissue was washed in triple distilled water, defatted in alcohol and dried by lyophilization. Extreme care was taken to avoid metal contamination of either liver tissue or serum at every point of handling.

Assessment of hepatic fibrosis

The clinical indices of hepatic fibrosis were evaluated histopathologically as well as by quantifying collagen content in the liver. All major liver function tests were also carried out in serum samples on the 7th, 14th and 21st days after administration of DMN. The paraffin-embedded blocks were cut into 5 mm sections and stained with hematoxylin and eosin. The stained sections were examined using a Nikon labophot microscope and photographed.

Collagen content in the liver tissue was measured as a biochemical parameter to assess the progression of fibrosis. Total collagen content in the liver tissue was determined by the estimation of hydroxyproline, a characteristic imino acid present in collagen. To estimate hydroxyproline, 100 mg wet liver tissue was hydrolyzed in 6 mol/L HCl in sealed tubes at 110°C for 16 h. The hydrolyzed samples were evaporated to dryness in a boiling water bath to remove acid, and the residue was redissolved in distilled water and made up to a known volume. It was treated with activated charcoal and filtered through Whatman filter paper. The clear filtrate was used for the determination of hydroxyproline according to the method of Woessner [15]. In brief, 1 mL of filtrate was mixed with 1 mL of freshly prepared chloramine-T solution and allowed to stand for 20 min. It was further mixed with 1 mL of 3.15 mol/L perchloric acid and left for 5 min.
Finally, 1 mL of freshly prepared p-dimethylaminobenzaldehyde was added and mixed well, and the mixture was incubated in a water bath at 60°C for 20 min. The absorbance of the solution was measured in a spectrophotometer at 560 nm.

Total collagen content in the liver tissue was calculated by multiplying the hydroxyproline content by a factor of 7.46 as described by George et al. [16].

All major liver function tests such as total bilirubin, alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (γ-GT) and albumin were carried out in the serum samples by conventional spectrophotometric methods.

Extraction of elements from the liver tissue

About 100 mg dried liver tissue was pre-digested with 2 mL of redistilled concentrated nitric acid in an extremely clean beaker with a cover glass at 110–120°C until it turned a pale yellow color. 2 mL of quartz-distilled concentrated perchloric acid (70%) was then added after cooling to ambient temperature and digested at about 180°C until it became a clear and colorless solution. It was cooled and made up to 10 mL with penta distilled deionized (Milli-Q18.2 MΩ) water (PDW) in order to obtain a final concentration of 10 mg dried liver tissue/mL. This was suitably diluted according to the concentration of a particular element in the liver and used for analysis by atomic absorption spectrophotometry (AAS). Two sets of blanks were similarly treated and incorporated in the assay.

Standards and sample preparations

During standards and sample preparations, all possible measures were taken to avoid contamination by elements at every processing and handling step. The standards were prepared and diluted in 50 mL screw capped polypropylene centrifuge tubes (Corning Life Sciences, Corning, New York). Prior to the preparation of standards, all tubes were rinsed with PDW and a pre-run was carried out for each element after appropriate instrumental settings. Tubes found contaminated by any element were discarded. Calcium and magnesium stock standards were prepared by dissolving Specpure calcium carbonate (2.497 g) and 99.99% pure magnesium metal strip (1.0 g) respectively, in a minimum volume of 1:4 diluted double distilled nitric acid and made up to 1 l with PDW. To obtain sodium and potassium standards, Specpure anhydrous salts (2.542 g and 1.907 g respectively) were directly dissolved in PDW and made up to 100 mL with PDW. Then it was diluted to 50-fold with PDW in order to obtain the lanthanum diluent.

Lanthanum diluent for calcium and magnesium

Lanthanum chloride was used as the diluent to remove the interference of anions such as phosphates and silicates, which bind calcium and magnesium and do not dissociate as free atoms in the flame. Lanthanum (La++) binds preferentially with phosphates and silicates, releasing calcium and magnesium for analysis [17]. The lanthanum diluent was prepared by dissolving 8.15 g lanthanum oxide (Specpure) in 33.5 mL of redistilled concentrated hydrochloric acid and made up to 100 mL with PDW. Then it was diluted to 50-fold with PDW in order to obtain the lanthanum diluent.

Analysis of elements

Analysis of essential elements present in serum and liver samples was performed by atomic absorption spectrophotometry. All the elements studied were analyzed on a Varian Techtron model AA-1475 (Varian Instrument Division, 611 Hansen Way, Palo Alto, California, USA) double beam atomic absorption spectrophotometer. Varian Techtron hollow cathode lamps were used for producing sharp resonance lines of the concerned element. Instrumental settings and analytical conditions employed for all elements studied are provided on Table 1. The serum was diluted 50-fold and liver extracts (10 mg/mL) 10-fold with lanthanum diluent for the determination of calcium. To determine the magnesium concentration, the serum was diluted 100-fold and liver extracts 20-fold with lanthanum diluent. Analysis of sodium was carried out after diluting the serum sample 1:10,000 with PDW in a two-step procedure in order to minimize the error during dilution. Liver extracts were diluted 50-fold with PDW. Samples used for the determination of potassium were prepared by diluting the serum 1:1000 and liver extracts 1:100 with PDW. The diluted samples were directly nebulized into the flame. All samples analyzed were in triplicate and the mean value was taken.

Statistical analysis

Arithmetic mean and standard error were calculated for the data. Results were statistically evaluated by using one-way analysis of variance (ANOVA). Mean control values were compared with experimental mean values on days 7, 14 and 21 using the least significant difference method. The

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Ca</th>
<th>Mg</th>
<th>K</th>
<th>Na</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength (nm)</td>
<td>422.7</td>
<td>285.2</td>
<td>766.5</td>
<td>589.0</td>
</tr>
<tr>
<td>Lamp current (mA)</td>
<td>3.5</td>
<td>3.5</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Slit width (nm)</td>
<td>0.5</td>
<td>0.5</td>
<td>1.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Fuel</td>
<td>Acetylene</td>
<td>Acetylene</td>
<td>Acetylene</td>
<td>Acetylene</td>
</tr>
<tr>
<td>Support</td>
<td>Nitrous oxide</td>
<td>Nitrous oxide</td>
<td>Air</td>
<td>Air</td>
</tr>
</tbody>
</table>
value of $P<0.05$ was considered as statistically significant. Pearson’s correlation coefficient was used to study the linear relationship between the alteration in levels of elements in the serum and liver beginning from control to day 21.

Results

Animal body weight and liver weight

The body weight of the experimental animals was monitored throughout the study. The DMN administered animals did not gain body weight during the course of treatment. A significant decrease ($P<0.001$) was noticed in the mean body weight of the animals on days 14 and 21 after the start of DMN administration. The liver weight of the experimental animals was also reduced significantly ($P<0.001$) on days 14 and 21 during DMN treatment (Fig. 1).

Clinical indices of hepatic fibrosis

The histopathological alterations of liver tissue during DMN-induced hepatic fibrosis and cirrhosis are shown in Figs. 2A–D. The control liver showed normal lobular architecture with central vein and radiating hepatic cords (Fig. 2A). Massive hepatic necrosis and collapse of the liver parenchyma were observed on day 7. The liver tissue also demonstrated severe centrilobular congestion, dilatation of central vein and sinusoids, with focal hemorrhage (Fig. 2B). Well developed fibrosis and early cirrhosis were present on day 14. There were multifocal hepatocyte necrosis and neutrophilic infiltration. Bridging necrosis and apoptosis were also present in certain
cases (Fig. 2C). On the 21st day, the liver sections demonstrated well developed cirrhosis with collagen fibers, intense neutrophilic infiltration and regeneration of hepatocytes (Fig. 2D).

The total collagen content in the liver tissue was significantly elevated on all the days measured with a maximum elevation of about 4-fold on day 21 (Fig. 3). The levels of total bilirubin, ALT, AST, ALP and \( \gamma \)-GT in the serum were significantly elevated and the albumin concentrations remarkably reduced (Table 2).

**Ascites**

Ascites were not present on any animals sacrificed on day 7. However, ascites was present in 40% of animals sacrificed on day 14 and 70% of animals sacrificed on day 21. The volume of ascitic fluid measured was from 10 to 60 mL. The maximum fluid accumulation was in day 21 group of animals.

**Serum and liver concentrations of calcium**

Calcium levels in the serum and liver tissue are demonstrated in Tables 3 and 4 respectively. A significant decrease \( (P<0.001) \) was observed in the serum calcium levels on the 14th and 21st days after the start of DMN administration. The difference was not significant on the 7th day. The maximum decrease in the serum calcium level was on the 21st day. A positive correlation \( (r=0.955) \) was noticed between the decreased calcium levels and reduced albumin concentrations in the serum (Table 5). A negative correlation was observed between the reduced serum calcium content and liver function tests except in the case of \( \gamma \)-GT (Table 5). An increase was noticed regarding calcium content in the liver on the 21st day after the start of DMN administration. The differences were not significant on any other days studied.

**Serum and liver concentrations of magnesium**

Magnesium concentrations in the serum were significantly \( (P<0.001) \) reduced on all the days measured after the start of DMN administration (Table 3). The depletion was gradual and remarkable. The maximum decrease of serum magnesium was on day 21 after the start of DMN administration and the value was about 50% less when compared to the mean control value. A positive correlation \( (r=0.951) \) was noticed between decreased magnesium and reduced albumin concentrations in the serum. The liver magnesium was decreased significantly on day 21 of DMN-induced hepatic fibrosis (Table 4).

**Serum and liver concentrations of potassium**

Potassium, the principal cation of intracellular fluid, reduced significantly in the serum and liver tissue on days 14 and 21 after the start of DMN administration. No significant alteration was noticed in the potassium level either in the serum or liver tissue on day 7. Reduced serum potassium and albumin levels demonstrated a highly positive correlation \( (r=0.999) \). Furthermore, negative correlations were observed between decreased serum potassium concentrations and increased liver function enzymes in the serum (Table 5). Hypokalemia contributes to the frequently recognized hypertension in experimental cirrhotic animals as well as in patients. Several lines of evidence suggest that potassium deficiency can increase blood pressure [8].

**Serum and liver concentrations of sodium**

Sodium, the principal cation in extracellular fluid, decreased in the serum on all the days measured during experimentally
induced hepatic fibrosis in rats (Table 3). The depletion was gradual with a maximum decrease on day 21 after the start of DMN treatment. A positive correlation was observed between decreased sodium and albumin concentrations in the serum ($r=0.964$). There was no significant alteration in liver sodium concentrations during DMN-induced hepatic fibrosis (Table 4). As in the case of potassium, correlation analysis demonstrated negative correlations between depleted serum sodium and liver function tests (Table 5).

**Discussion**

Dimethylnitrosamine-induced hepatic fibrosis in rats is a reproducible and potentially valuable animal model for studying the pathogenesis of human hepatic fibrosis and cirrhosis. The 21-day course of DMN administration in rats produced centrilobular necrosis and well-developed fibrosis, as present in alcoholic liver diseases. The model has been evaluated previously and demonstrated that it is an excellent animal model for studying the biochemical and pathophysiological as well as molecular alterations associated with the development of hepatic fibrosis and cirrhosis [18,19].

The results of the present investigation suggest that alterations of essential elements play a role in the aggravation of DMN-induced hepatic fibrosis in rats. Reports are not available or scanty regarding concentrations of minerals in experimental liver fibrosis or cirrhosis. Many factors interfere with mineral metabolism in fibrotic animals. Some of the factors are gastrointestinal disturbances, malabsorption and interactions between elements. It is important to note that the animal body weight and liver weight were significantly reduced on days 14 and 21 after the start of DMN administration (Fig. 1). The remarkable decrease of both serum and liver ascorbic acid concentrations reported [20] in DMN-induced hepatic fibrosis in rats may also have a relationship with the alterations of minerals.

The precise mechanism of disturbed mineral metabolism in DMN-induced hepatic fibrosis is not clear. The results of the present study suggest that alteration in minerals during DMN-induced hepatic fibrosis is secondary to the disease process and the altered minerals may further deteriorate the situation. The decreased albumin synthesis by the fibrotic liver contributes towards the reduction of serum calcium and magnesium concentrations. Similarly, ascites plays an important role in the depletion of serum electrolytes in liver cirrhosis [21].

Significantly decreased serum calcium levels in patients with liver cirrhosis have been reported [22]. A reduction in platelet calcium ion was also reported in patients with liver cirrhosis [23]. Furthermore, calcium deficiency was reported in alcoholics due to malnutrition and calcium imbalance [24]. In the present investigation also, serum calcium concentration was significantly decreased on days 14 and 21 after the start of DMN administration (Table 3). The exact mechanism of decreased serum calcium concentrations in DMN-induced hepatic fibrosis is not clear. The marked decrease of serum albumin levels (Table 2) observed in the present investigation may be partly responsible for the reduced serum calcium levels. About 47% of serum calcium is bound to proteins, especially albumin. It was reported that the principal pathogenesis of hepatic osteodystrophy is due to the intestinal calcium malabsorption due to lower serum albumin concentrations [25]. Furthermore, a significant decrease was reported in plasma levels of 1α, 25-dihydroxyvitamin D in patients with liver cirrhosis [26,27]. Dihydroxyvitamin D is responsible for the retention and resorption of calcium ions by the kidney tubules.

**Table 4**
Calcium, magnesium, potassium and sodium levels in the serum during DMN-induced hepatic fibrosis in rats

<table>
<thead>
<tr>
<th>Elements</th>
<th>Control ($n=16$)</th>
<th>Day 7 ($n=16$)</th>
<th>Day 14 ($n=12$)</th>
<th>Day 21 ($n=10$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium (μg/mL)</td>
<td>110.15±3.92</td>
<td>103.75±3.75</td>
<td>91.50±3.83**</td>
<td>82.58±3.59**</td>
</tr>
<tr>
<td>Magnesium (μg/mL)</td>
<td>22.32±1.05</td>
<td>16.38±0.98**</td>
<td>13.30±1.20**</td>
<td>11.31±0.65**</td>
</tr>
<tr>
<td>Potassium (mg/100 mL)</td>
<td>28.50±1.19</td>
<td>26.16±0.97</td>
<td>22.42±1.06**</td>
<td>19.75±0.93**</td>
</tr>
<tr>
<td>Sodium (mg/100 mL)</td>
<td>354.58±8.76</td>
<td>312.92±10.68*</td>
<td>289.66±11.99**</td>
<td>270.16±12.17**</td>
</tr>
</tbody>
</table>

All values are expressed as μg/100 mg liver dry weight.
Values are mean±standard error.
* $P<0.01$ (by ANOVA).
** $P<0.001$ (by ANOVA).
The role of calcium ions in collagen metabolism has been well established [28]. Calcium is essential for optimum activity of MMP-1, MMP-8 and MMP-13 (collagenase-1, 2 and 3 respectively), the primary native collagen degrading enzymes [29–31]. An increase in the level of intracellular free calcium in hepatic stellate cells as a result of transmembrane Ca²⁺ influx that is mediated through transforming growth factor-β1 (TGF-β1) has been reported [32]. Hepatic stellate cells play the key role in liver fibrosis and TGF-β1 is the key factor that stimulates collagen production by stellate cells. The increased intracellular calcium mobilization by TGF-β1 may be involved in increased collagen biosynthesis in hepatic fibrosis.

Magnesium is one of the most important micronutrients and plays a vital role in the immune system, in both innate and acquired immune response [33]. A significant decrease in either plasma or serum magnesium level has been reported in patients with liver cirrhosis [22,34,35]. Decreased plasma magnesium level has also been reported in thioacetamide-induced liver cirrhosis in rats [36]. The exact mechanism of the marked decrease of serum magnesium concentration observed in the present investigation is not clear. This could be due to the malabsorption or impaired tubular resorption. It was reported that decreased tubular resorption and impaired acid-base balance.

May be attributed to severe ascites associated with the pathogenesis of the disease, decreased tubular resorption and impaired acid-base balance.

Significant decrease of serum sodium concentrations has been reported in liver cirrhosis [5–7]. Hyponatremia is an inevitable phenomenon and an excellent predictor of outcome in patients with advanced cirrhosis [45,46]. In the present study also, serum sodium concentration was significantly reduced on all the days measured after the start of DMN administration. Decreased serum sodium in hepatic fibrosis is mainly due to ascites, the excessive retention of free water by the kidney [44,47]. It was reported that hyponatremia is not only by an excess of total body water but also by a deficit of serum potassium [21]. The most probable cause of depleted serum sodium in DMN-induced hepatic fibrosis is due to retention of excess water.

In conclusion, results of the present study suggest that DMN-induced hepatic fibrosis and cirrhosis play certain role in mineral disturbances. The acute centrilobular and perisinoidal (immunologically mediated) hepatic necrosis can be the primary cause of mineral abnormalities. Low levels of albumin and related ascites may be one of the major causes of the imbalance of mineral metabolism in liver diseases. The overall pathophysiological basis of mineral disturbances in hepatic fibrosis is far more complex and further studies are needed.

Acknowledgments

This work was supported by the Indian Council of Medical Research, New Delhi by grant (No. 3/1/2/3/(9201540)/92-NCD-III) to the author. The author is thankful to the Director of the Central Leather Research Institute (Council for Scientific and Industrial research), Chennai for providing facilities to carry out this work. The author is also grateful to the Director of the Central Leprosy Teaching and Research Institute (Government of India), Chengalpattu, Tamil Nadu, for his permission to use the Atomic Absorption facility at the institute.

References
