A study of histopathological effects and functional tests in liver and kidney of laboratory male mice treated with ammonium chloride

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Abstract

The present study was aimed to investigate the effects of ammonium chloride on mice. The animals were divided into three groups: groups 2 and 3 were treated orally with ammonium chloride at doses of 2 and 4 mg/body weight, respectively for a period of three weeks, while group 1 served as a control group. The tissue sections which were made from mice organs (liver and kidney) which treated with 4mg/ body weight of ammonium chloride proved that this dose has toxic effect only on the liver and kidneys. In the liver, the histopathological effects are represented by hypertrophy and irregular shape of nucleus, degeneration of cytoplasm, congestion of sinusoid, bleeding and infiltration of inflammatory cells. In kidneys, the effects focus on renal tubules only which are represented by the degenerative changes, necrosis and infiltration of inflammatory cells. This study was also carried out to investigate the influence of ammonium chloride on levels of urea, creatinine, total protein, lipid (triglyceride and cholesterol), and liver marker enzymes such as AST, ALT and ALP. Oral administration of ammonium chloride (4mg/body weight) caused a significant increase in the levels of AST and a significant decrease in the level of ALP and total protein in mice. Treated mice with ammonium chloride at a dose of 2 and 4mg/ body weight for 21 days showed a significant decrease in levels of creatinine, triglyceride and cholesterol, while ALT and urea had no affect at two doses of ammonium chloride. In conclusion, ammonium chloride causes direct hepatotoxicity and nephrotoxicity.

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Introduction

Ammonium chloride is a reagent that is used in a variety of industrial and research applications. Industrial uses include electroplating, tinning, and manufacture of dyes. It is also used as a fluxing agent for the galvanizing of steel, the refinement of zinc and the coating of sheet iron with zinc (Williams, 2006). Ammonium chloride acidifies the urine and is used to dissolve certain types of bladder stones; it may also be used to promote the excretion of certain toxins or drugs into the urine. It may be amended with certain antibiotics such as tetracycline and penicillin to increase their effect against certain bacteria in the urine (Foster and Smith Pharmacy, 2010).

In biological research, ammonium is often used for lyases of human red blood cells (Kang et al., 2002). Also, it is used in the isolation of proteins from 50S ribosomal subunits of Bacillus stearothermophilus (Gewitz, 1987). In mammals, ammonia is an important nitrogen substrate in several reactions and plays an important role in nitrogen homeostasis of mammalian cells. Ammonia is produced by amino acid and protein catabolism and is toxic to brain and muscles. Ammonia toxicity results in free-radical generation that leads to oxidative stress and tissue damage (Lena and Subramanian, 2004).

Ammonia affects both excitatory and inhibitory synaptic transmission in the mammalian brain through a variety of mechanisms (Monfort and Felipo, 2005). It is the major metabolic end product during the catabolism of proteins. Because of its high toxicity, even at a very low concentration in vivo, ammonia is either excreted directly or converted to some less toxic compounds such urea, uric acid or amino acids (Cooper and Plum, 1987; Randall and Wright, 1987; Campall, 1991; Wood, 1993). Ammonium is also an endogenous substance that serves a major role in the maintenance of the acid-base balance (Hall, 2015).

Ammonia is converted to urea in the liver by urea-cycle enzymes, which is then excreted by the kidneys. Hyperammonemia may result from genetic defect or deficiency of the urea-cycle enzymes or from acquired condition such as Reyes syndrome, liver failure, high-dose chemotherapy and severe infection (Treem, 1994).

It has been well established that in man, rat and dog urinary ammonia increases in response to metabolic acidosis (Pollak et al., 1965).

Ammonia toxicity occurs partly via oxidative stress, which leads to lipid peroxidation and free radical generation. This causes hepatic dysfunction and failure, which is a primary cause of neurological disorders and alteration in the function of central nervous system associated with hyperammonemia, such as hepatic encephalopathies, Reye syndrome, irritability, somnolence, vomiting and derangement of cerebral function, coma and death (Harikrishnan et al., 2008; Vijayakumar and Subramanian, 2010).

In the present study, the effects of ammonium chloride on mice liver and kidney are investigated. However, no available literatures dealing with histological changes and function tests in liver and kidney evoked by ammonium chloride administration.

Materials and methods

Test material

Ammonium Chloride NH\textsubscript{4}Cl powder (1kg) product of (Reagent Chemical Services Company Ltd. UK). This powder was dissolved in distilled water.

Animals

Forty-eight adult male albino mice Mus musculus L. weighting 23-25 gm were used for this study and were obtained from the animals house of Department of Biology, College of Education, University of Basrah. Animals were housed in cages (4 animals/ cage) and were fed with standard diet for 21 days at room temperature (25±3°C) with controlled light-dark cycle throughout the experiment (Jawad, 1996).

Experimental Design

Animals were divided into three groups comprising
eight animals in each group as follows:

Group 1: Mice were served as control treated with 0.2 ml of distal water.

Group 2: Experimental animals were orally given 0.2 ml of ammonium chloride solution at a dose level of 2 mg/ body weight daily, for a period of three weeks.

Group 3: The animals were orally given 0.2 ml ammonium chloride solution at a dose level of 4 mg/ body weight daily for a period of three weeks.

**Histopathological examination**

For light microscopic examination, liver and kidney tissues from each group were fixed with Bouin’s solution and embedded with paraffin. After routine processing, paraffin sections of each tissue were cut into 7 μm thickness and stained with hematoxylin and eosin (Humason, 1972).

**Biochemical Analysis**

At the end of experimental period, mice were anaesthetized with ether. Blood samples were withdrawn by heart puncture and serum was separated, centrifuged at 3500 rpm for 15 minutes, and blood serum was then collected and stored at 4 °C prior immediate determination of serum parameters. Serum aspartate transaminase (AST) and alanine transaminase (ALT) were determined according to the methods of Reitman and Frankel (1957). Serum alkaline phosphatase (ALP) and urea were determined according to the methods of King and King (1954) and Wills and Savory (1981), respectively. Serum creatinine and total protein were estimated using the methods of Butch (1994). Serum total cholesterol and Triglycerides were determined according to the methods of Tietz (1995). All of these parameters were measured using spectrophotometer. Kits were provided by Biolabo and Biomerieux Company (France).

**Statistical Analysis**

The results of biochemical tests were analyzed using the Statistical Package for Social Sciences (SPSS for windows, version 12.0). Comparisons were made between experimental groups using one-way analysis of variance (ANOVA) followed by Dennett’s test. Values of less than 0.01 were regarded as statistically significant.

**Results**

**Histopathological results**

The histopathological examination of the liver of group 1 and 2 shows normal tissues which included the center vein, hepatocytes arranged as cords and between them sinusoids.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>AST (IU/L)</th>
<th>ALT (IU/L)</th>
<th>ALP (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group (Distal water)</td>
<td>9.63 ± 0.41</td>
<td>7.61 ± 0.49</td>
<td>128.61 ± 2.51</td>
</tr>
<tr>
<td>Ammonium chloride (2mg/ body weight)</td>
<td>9.12 ± 0.46</td>
<td>6.61 ± 0.24</td>
<td>120.06 ± 3.45</td>
</tr>
<tr>
<td>Ammonium chloride (4mg/ body weight)</td>
<td>*12.21 ± 0.89</td>
<td>7.92 ± 0.23</td>
<td>*103.15 ± 2.74</td>
</tr>
</tbody>
</table>

* Significant differences (p< 0.01 & 0.05) compared with the control group.

Histological examination of liver of group 3 (treated with high dose) revealed histopathological alterations which included hypertrophy and irregular shape of nucleus with prominent central nucleoli, massive degeneration and in other animals, the hepatocytes had numerous pale vacuoles of widely different sizes. Other hepatocytes were necrotized. Liver sections reflected other signs of injury as indicated by congestion of sinusoid; bleeding and dilatation of infiltrations by large mass of leukocytic inflammatory cells were observed. These inflammatory cells include lymphocytes, giant multinucleated cell and cluster of epithelioid histiocytes (Fig. 1 and 2).
Table 2. Effect of ammonium chloride on function tests in kidney of mice.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Urea (mg/dL)</th>
<th>Creatinine (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group (Distilled water)</td>
<td>33.45 ± 1.31</td>
<td>0.95 ± 0.03</td>
</tr>
<tr>
<td>Ammonium chloride (2mg/ day)</td>
<td>32.11 ± 1.25</td>
<td>0.47* ± 0.04</td>
</tr>
<tr>
<td>Ammonium chloride (4mg/ body weight)</td>
<td>37.38 ± 1.47</td>
<td>0.34* ± 0.04</td>
</tr>
</tbody>
</table>

* Significant differences (p< 0.01 & 0.05) compared with the control group.

Examination of tissue section of kidney of group 3 (treated with high dose) revealed histopathological changes on lining epithelial tissue of renal tubule which was represented by degenerative changes, dilation of lumen of distal convoluted tubule and lysis of the apical cell membrane of the proximal convoluted renal tubule and seems that these tubules have a broad lumen and its lacks the brush boarder in comparison with the control group. Kidney tissue sections showed atrophy cells of renal tubule and deformation of nuclei of other epithelial cells of both distal and proximal convoluted renal tubule. In addition, the bleeding was observed between cortical renal tubule and infiltration of inflammatory cells near large vessel (Fig. 2 and 3).

Biochemical results

The results of this study showed a significant increase in aspartate transaminase enzyme level at p<0.01 and p< 0.05 for male mice treated with the high dose (4 mg) of ammonium chloride, and a significant decrease in alkaline phosphatase with high dose only compared with the control group, while alanine transaminase level in male mice was not affected by the treatment with two doses in comparison with the control group as shown in table (1). The results also showed a significant decrease in serum creatinine level of mice treated with both doses (2 and 4 mg). The present data showed no significant effect on serum urea with the two doses as shown in table (2). A significant increase in serum triglyceride and cholesterol levels of male mice treated with two doses (2 and 4 mg) of ammonium chloride, and a significant decrease in total protein level with high dose only compared with the control group (Table 3).

Table 3. Effects of ammonium chloride on proteins and lipids level of mice.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Total Protein (g/dL)</th>
<th>Triglycerides (mg/dL)</th>
<th>Cholesterol (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group (Distilled water)</td>
<td>7.75 ± 0.34</td>
<td>158.15 ± 3.01</td>
<td>150.72 ± 2.35</td>
</tr>
<tr>
<td>Ammonium chloride (2mg/ body weight)</td>
<td>7.19 ± 0.28</td>
<td>190.40* ± 3.45</td>
<td>163.75* ± 3.41</td>
</tr>
<tr>
<td>Ammonium chloride (4mg/ body weight)</td>
<td>5.96* ± 0.25</td>
<td>196.21* ± 2.76</td>
<td>169.89* ± 2.89</td>
</tr>
</tbody>
</table>

* Significant difference (p< 0.01 & 0.05) compared with the control group.

Discussion

Ammonia is presented in all living organisms as a product of degradation of protein and other nitrogenous compounds (Hall, 2015). At higher levels, ammonia is toxic, leading to functional disturbances in the central nervous system that could lead to coma and death (Kosenko et al., 1998). To avoid the deleterious effects of ammonia, ureotelic animals detoxify ammonia by incorporating it into urea that is eliminated in urine (Nelson et al., 2008). The liver is the most important site of ammonia metabolism; it removes the toxic ammonia into urea in periportal hepatocytes and/or as glutamine in perivenous hepatocytes (Nelson et al., 2008; Subash and...
Subramanian 2009). By doing so, the liver also plays a major role in the metabolic regulation of systemic pH, because hydrogen ions released from NH₄ during the synthesis of neutralize excess bicarbonate produced by the breakdown of amino acid. An increase level of circulatory ammonia might indicate a hyperammonemic condition in rats treated with ammonium chloride (Lena and Subramanian, 2004; Essa and Subramanian, 2007). Steven et al. (2009) showed that the damage of hepatocytes in liver increased the level of ammonia in blood and caused hyperammonemic condition due to incapability of hepatocytes on synthesis of the urea, and all of this leads to decrease of urea in blood.

Fig. 1. (A) Liver of the control group (400x), (E&H). (B) Liver of treated group (4mg ammonium chloride) showing hypertrophy of nuclei (arrow) (400x), (E&H). (C) Liver of treated group (4mg ammonium chloride) showing irregular shape of nucleus in hepatocyte with prominent central nucleoli (arrows) (400x), (E&H). (D) Liver of treated group (4mg ammonium chloride) showing massive degeneration (thin arrows) and necrosis (thick arrows) (400x), (E&H). (E) Liver of treated group (4mg ammonium chloride) showing vacuole degeneration (thin arrows) and congestion of sinusoid (thick arrows) (100x), (E&H). (F) Liver of treated group (4mg ammonium chloride) showing increase of giant multinucleated cells (arrow) and infiltration (stars) (400x), (E&H).

The present study the histological examination of liver and kidney of treated mice with ammonium chloride showed many histopathological changes which included hypertrophy and irregular of nucleus, massive degeneration, necrosis and increase of giant cells, and congestion of sinusoid in liver. Liver is one of the most important organs of the body which is sensitive to toxins being a member of the President metabolism of many toxic substances entering the body of the organism. So, it may exhibit severe histopathological effects than those toxins and exceed the limits of its ability to get rid of them, which may explain the diversity of histopathological changes in the liver and increase the intensity (Klaassen and Watkins, 1999). The ammonium chloride in mice may cause changes in the functions responsible for removing toxins so reflected on the liver’s ability to remove the harmful effects of ammonium chloride.
system cells (Rubin and Reisner, 2014). Some studies suggest that exposure to toxins cause a degeneration of the liver cells to failure in protein representation and its accumulation (Thophon et al., 2003) or due to the inhibition of toxic substances the process of protein synthesis through its effect on the enzyme protein phosphatase (Stevens et al., 2009). This may be considered as one of the reasons for the occurrence of necrosis. The vacuolar degeneration in liver may be represented by the fatty changes which may be developed into fatty necrosis (Rubin and Reisner, 2014). This finding is in agreement with Subash and Subramanian (2009) who reported that 100 mg/kg body weight; i.p. ammonium chloride rats show liver fibrosis, steatosis and sinusoidal dilatation. The gavage studies in rats have shown some lesions of gastric mucosa with notable histopathological effects (Takeuchi et al., 1995; Mori et al., 1998). In the kidney, the pathological changes showed no damage in the glomeruli and the changes are focused in the renal tubule, because of the structural nature of the glomeruli which restrict the movement of blood cells and most large molecules opposite that allow to many substances (including water and solutes) in the blood pass through the renal corpuscle and enter the renal tubule (Hall, 2015). The histopathological changes of hyperammonemic mice are infiltration of inflammatory cells, deformation and hypertrophy of nucleus. The necrosis of the lining of the renal tubule possibly causes later renal failure, especially when number of affected tubule grows (Cotran et al., 1999).

**Fig. 2.** (G) Liver of treated group (4mg ammonium chloride) showing bleeding and infiltration of inflammatory cells (200x), (E&H). (H) Liver of treated group (4mg ammonium chloride) showing congestion of the sinusoid (400x), (E&H). (I) Kidney of control group showing cortical renal tubule (400x), (E&H). (J) Kidney of control group showing the medullary renal tubules (400x), (E&H). (K) Kidney of treated group (4mg ammonium chloride) showing deformation of nucleus (400x), (E&H). (L) Degeneration (arrow) and dilation in distal convoluted renal tubule (stars) of treated group (4mg ammonium chloride) (400x), (E&H).
Oxidative stress injury is actively involved in the pathogenesis of ammonium chloride and induced acute kidney injury. Reactive oxygen species (ROS) directly act on cell components, including lipids, proteins, and DNA, and destroy their structure. Increased levels of circulatory ammonia in mice treated with ammonium chloride may be due to the liver damage caused by ammonia-induced free radical generation. Reports have shown that excess ammonia induces nitric oxide synthase, which lead to the enhanced production of the nitric oxide, leading in turn to oxidative stress and liver damage (Kosenko et al., 2000; Schliess et al., 2002).

The present study showed also a significant increase in aspartate transaminase enzyme (AST) at high dose (4 mg ammonium chloride/ day) of treated group with ammonium chloride, and significant decrease in alkaline phosphatase with high dose only compared with control group. The alanine transaminase level in two treated groups was not affected by treatment as compared with control (Table 1). The increased activities of these serum markers observed in this study correspond to considerable liver damage induced in ammonium chloride mice. Moreover, the estimation in serum is useful quantitative marker to indicate hepatocellular damage (Sallie et al., 1991). Serum AST, ALT and ALP are the sensitive markers employed in the diagnosis of liver diseases. When the liver cell plasma membrane is damaged, numerous enzymes normally located in the cytosol are released into the blood stream (Rajesh and Latha, 2004).

Furthermore, the present study showed a significant decrease in serum creatinine level of mice treated with both doses (2 and 4 mg ammonium chloride) as shown in table (2), and no significant effect in serum urea with the two doses, besides a significant decrease in total protein level with high dose compared with the control group. The decreasing of serum creatinine may be due to the decreasing of serum total protein. The free radicals affect the activity of skeletal muscles.
and thereby cause decreasing of serum creatinine (Kumar et al., 2010). Also, they cause oxidation of molecular protein in serum and this may result in decreasing the total protein in serum (Ujjwal and Dey, 2010). Mostafa et al. (2009) mentioned that the free radicals caused a significant decrease in plasma proteins.

Previous reports stated that ammonium (chloride/acetate) salt induces ammonia toxicity party via oxidative stress, which leads to lipid peroxidation and free-radical generation (Essa and Subramanian, 2006). Moreover, the excess of ammonia intoxication leads to excessive activation of N-methyl-D-aspartate (NMDA) receptors leading to neuronal degeneration and death (Subash and Subramanian, 2010). Also, the elevated levels of circulatory liver-markers and lipid peroxidation products in ammonium chloride in rats might be due to the liver damage caused by ammonia-induced free radical generation (Essa and Subramanian, 2006; Thenmozhi and Subramanian, 2011).

In the present study, the administration of ammonium chloride caused a significant increase in the levels of serum lipids (cholesterol and triglycerides as indicated in Table (3). These findings indicate that hyperammonemia may be accompanied by complication of atherosclerosis. Some studies showed that the levels of serum and tissue lipid were elevated during hyperammonemic conditions (Lena and Subramanian, 2004; Lena and Subramanian, 2004; Essa et al. 2010; Subash and Subramanian, 2012). It has been reported that ammonium (chloride/acetate) salts may deplete levels of α-KG and other Krebs cycle intermediates (Lena and Subramanian; 2004; Essa and Subramanian, 2006) and thus elevate the levels of acetyl coenzyme A. This acetyl Co A may be used for the synthesis of fatty acids and cholesterol, since fatty acids of different sources are used as substrates for synthesizing triglycerides and phospholipids. The elevated levels of acetyl CoA may increase levels of lipid profile (free fatty acids, triacylglycerols, phospholipids and cholesterol). Another important function of α-KG occurs in the formation of carnitine (Vijayakumar and Subramanian, 2010). Carnitine acts as a carrier of fatty acids into cell mitochondria so that proper catabolism of fats can proceed.

**Conclusion**

In conclusion, ammonium chloride causes direct hepatotoxicity and nephrotoxicity. The results indicate that the exposure of male mice to ammonium chloride affects both liver and kidneys, and this may be due to the generation of free radicals by showing their toxicity on liver and kidneys.

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