Histol Histopathol (2011) 26: 883-892

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Histopathological changes in gerbil liver and kidney after aluminum subchronic intoxication

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Summary. Young gerbil livers and kidneys were analyzed by means of light and electron microscope to assess the histopathological changes caused by prolonged systemic aluminum (Al) administration. The experimental group was injected with $AlCl_3$ i.p. for 5 weeks, while litter mates received PBS as sham-injected controls or served as untouched controls. Mortality occurred in 33% of experimental and 12.5% of sham-injected groups. The animals were perfused intracardially with 1% glutaraldehyde plus 1% paraformaldehyde and samples of liver and kidneys were processed for aluminum and iron histochemistry and conventional light- and transmission electron microscopy. White deposits composed of cellular debris appeared on the surface of liver and kidneys and in the mesentery as a consequence of Al treatment. Adherences of Glisson capsule to the diaphragm, as well as scattered small foci of hepatocyte necrosis with non-caseificant microgranulomas and mild portal inflammation, developed in the experimental group. Sham-injected animals also exhibited these granulomas but to a lesser degree. Al deposits were found in experimental animal granulomas and inside macrophages cytoplasm scattered throughout the liver. Iron deposition appeared in pericentral hepatocytes of experimental animals, in granulomas and in portal spaces of the three groups of animals. Ultrastructurally, hepatocytes of experimental animals showed mitochondria hyalinization, disintegration of endoplasmic reticulum and clustering of ribosomes. Phagolysosomes appeared larger and occurred more frequently in both hepatocytes and Kupffer cells of experimental animals. In 2 out of the 6 experimental animals studied, tubular atrophy was present in the renal cortical region, the kidneys of the remaining animals appearing normal. Al and iron were found very occasionally in the kidney parenchyma of experimental animals, while isolated mesangial cells showed iron deposits in a few glomeruli of both experimental and the two groups of control animals.

Key words: Aluminum, Liver toxicity, Kidney toxicity

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Introduction

Aluminum is a trace element in mammals, for which, unlike plants, no known biochemical function has so far been described. Al compounds are insoluble in water at neutral pH, and although Aluminium (Al) is the most abundant metal in the earth's crust (Macdonald and Martin, 1988) the amount in food is slight. People have been calculated to ingest between 3 and 5 mg per day, of which only 0.8% is actually absorbed (Greger and Baier, 1983). This amount is far from what is considered the safe limit of 125 mg daily uptake, but the risk of intoxication arises when widely-used Al-containing pharmaceuticals are taken, since these may extend the daily intake of Al to hundreds or even thousands of milligrams. Among medicines containing Al are gastrointestinal protectors, anti-diarrhea drugs, phosphate binders, buffered aspirins, vaccines and allergen injections (Lione, 1985). Other medical situations may also give rise to Al intoxication, such as inadvertent Al contamination of infusion solutions or dialysate, after administration of total parenteral nutrition, in patients with severe burns, following alum irrigation to prevent urinary bladder bleeding and during cranial bone cement surgery (Alfrey et al., 1976; Galle et al., 1980; Wilhelm et al., 1987).

Chronic Al intoxication may cause Vitamin D refractory osteodystrophy, hypochromic microcytic anemia and progressive encephalopathy, leading to death in months or years. Al induces stress proteins expression

(Stacchiotti et al., 2006) as well as oxidative stress (Prakash and Kumar, 2009), shows genotoxicity (Balasubramanyam et al., 2009), alters iron homeostasis (Ward et al., 2001) and has been proven to enhance the aging process (Kaur et al., 2003; Stacchiotti et al., 2008). It has also been involved in Alzheimer disease, Parkinson-dementia complex of Guam, Down syndrome, Wilson's disease and amyotrophic lateral sclerosis (Flatten, 1990; Sedman, 1992; Walton, 2007). Additional pathologies, such as pulmonary fibrosis, spinocerebellar degeneration and skin telangiectasias, have been reported after a high level of Al occupational exposure, and epidemiologic studies have revealed a higher incidence of lung and bladder cancer (Thériault et al., 1984). Moreover, granulomas and non-specific panniculitis have been described in Al-containing injection sites (Valtulini et al., 2005; Chong et al., 2006).

Although different aspects of Al toxicokinetics have already been studied (Wilhelm et al., 1990; Bharathi et al., 2008), data regarding Al distribution and toxicity is controversial because of the different methods and species utilized. A previous experimental study has reported that after systemic injections Al is accumulated particularly in the kidneys and liver (Forrester and Yokel, 1985); however this is accompanied by no histopathological data. Further studies have provided some information about liver or kidney pathology associated to Al in hemodyalized patients (Galle et al., 1987), following experimental Al intoxication in rabbits (Bertholf et al., 1989) and rats (Ebina et al., 1984; Stacchiotti et al., 2006), and in Al phosphide poisoning in humans (Sinha et al., 2005), contrasting with the absence of significant changes reported in the liver after experimental chronic intoxication in rats (Somova et al., 1997). Thus, in view of the controversial pathology involved in Al intoxication and as a result of the fact that the liver and kidney are two organs of primary interest in toxicology, more attention should be paid to their histological aspects, with a special focus on iron homeostasis alteration. In the present paper, we intend to contribute to a better understanding of the potential risks of Al intake and administration after prolonged systemic exposure to this metal, by showing, for the first time, the histological changes appearing in gerbil liver and kidneys after Al subchronic intoxication.

Materials and methods

A total number of 22 young male gerbils aged 3 to 3.5 months (62.5 g to 73.5 g) were used at the beginning of the experiment. Animals were kept air-conditioned at 20-22°C, 50% humidity and 12/12 h light/dark cycle. Care and manipulation of the animals followed the guidelines of the European Communities Council (86/609/EEC) for laboratory animal care and experimentation. A group of 9 animals were injected intraperitoneally with 10.4 μmol of $AlCl_3$/100 g of body weight (water solution warmed to 37°C) for 5 days a week over a period of 5 weeks, so that, approximately, the total amount of Al administered was 5 mg. Another group of 8 litter mates were treated with phosphate buffer saline (PBS, pH=7.4 warmed to 37°C) in an i.p. dose of 1 ml/100 g of body weight, during the same period, serving as sham-injected controls. Finally, a group of 5 litter mates were used as untouched controls. After the experiment, surviving animals were anesthetized with Equitesin and fixed by intracardial perfusion with 1% glutaraldehyde plus 1% paraformaldehyde in a phosphate buffer pH=7.4. Samples of liver and kidney were rinsed in a phosphate buffer and processed for light and electron microscopy. For light microscopy, pieces were embedded in paraffin wax and stained with hematoxylin and eosin (H&E), Masson's trichrome, PAS, the solochrome azurine method for aluminum and Perl's Prussian blue for iron. Samples for transmission electron microscopy were post-fixed in osmium tetroxide and processed conventionally.

Results

Mortality

Three animals from the experimental group and one from the sham-injected group died before the experiment was finished, representing one third and 12.5%, respectively, of mortality in their groups. These animals were not considered for the study, so that the final number of subjects for analysis was 6 experimental animals, 7 sham-injected and 5 untouched controls.

Liver

Macroscopically, multiple white deposits were found in the peritoneum, mesentery and on the liver surface in the experimental animals; these deposits were composed of cellular debris, as was revealed by light microscopy. Sham-injected and untreated control animals did not show any of these deposits. Externally, in the experimental animals the Glisson capsule developed adherences to the diaphragm epimysium and, in the second half of the experiment, four of these experimental animals showed signs of clinical peritoneal irritation, visible in the presence of collected liquid in the intraperitoneal space, and abdominal hypersensitivity when manipulated for injection. Microscopically, in these four animals the Glisson capsule appeared markedly thickened due to an accumulation of a poorly vascularized granulation tissue focally invading the underlying liver parenchyma. This granulation tissue appeared rich in lymphocytes with an admixture of macrophages, some of them giant cells (Fig. 1), and extended into the major intrahepatic branches of the capsular connective tissue, where it surrounded large foci of amorphous PAS-positive, slightly eosinophilic masses.

The experimental animals also showed, with light microscopy, a mild chronic inflammatory infiltrate in the

portal spaces (Fig. 2) and some irregularly dispersed small foci of hepatocyte degeneration and necrosis. Lymphocytes and monocytes accumulated in these foci forming non-caseificant microgranulomas (Fig. 3), whereas their neighboring hepatocytes showed signs of degeneration, including loss of their glycogen content, as seen by absence of PAS-positive reaction. By means of solochrome azurine histochemical reaction, intracellular

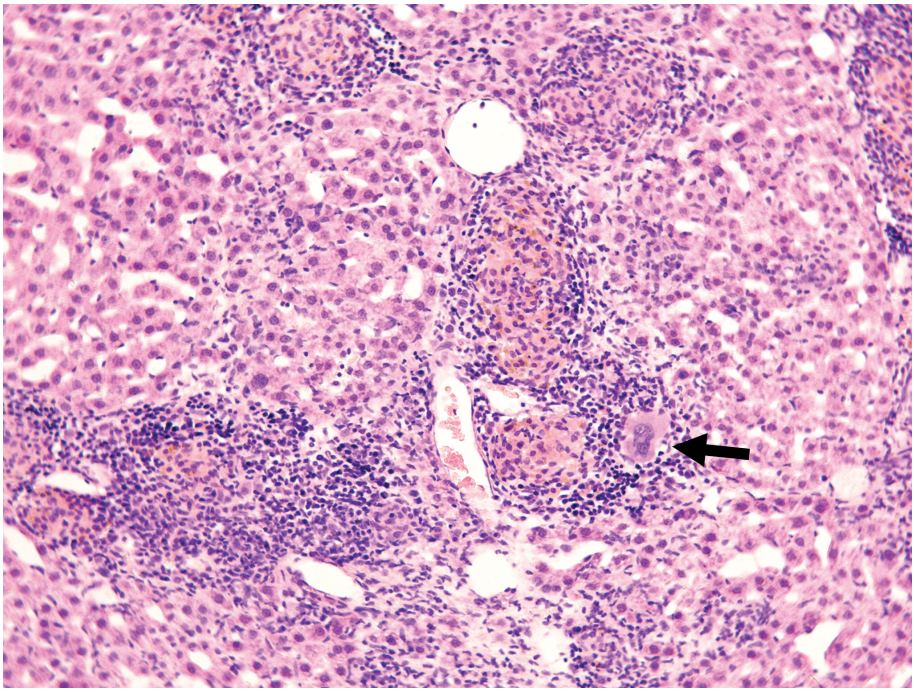

Fig. 1. Histological section of liver stained with hematoxylin and eosin in an experimental animal. Chronic hepatitis with non-caseificant granulomas were seen in hepatic parenchyma. Note the presence of giant cells (arrow). x 240

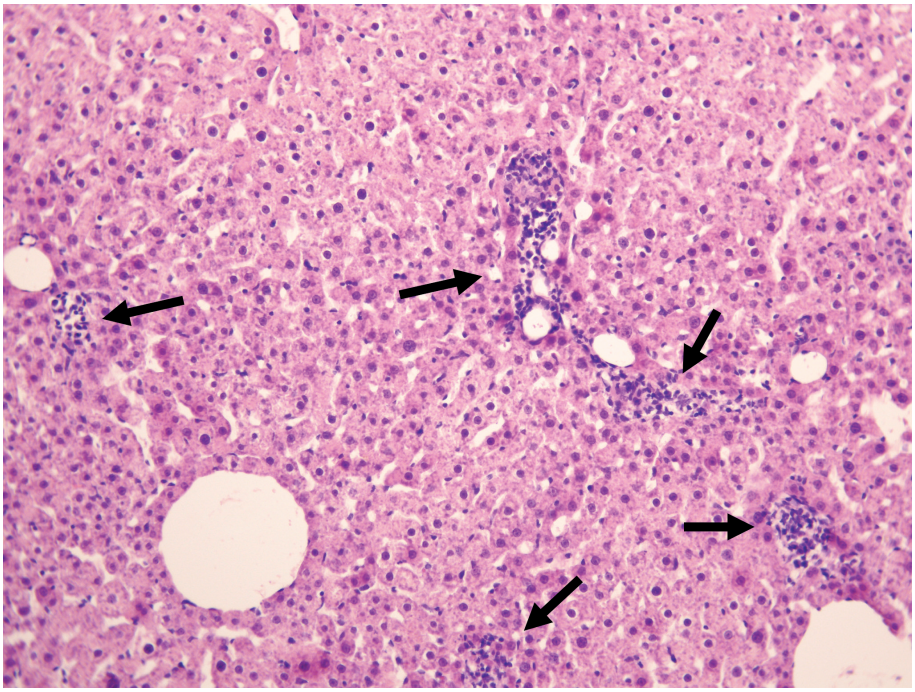

Fig. 2. Histological section of liver stained with hematoxylin and eosin in an experimental animal. Chronic periportal inflammation (arrows) occurred throughout the liver. x 240

deposits of Al were seen to be filling the connective tissue surrounding the liver and in the peritoneum, as well as inside degenerative hepatocytes forming microgranulomas and in macrophage cytoplasm (Fig. 4). Granulomatous hepatitis and portal inflammation were also seen in sham-injected animals but to a lesser degree, while granulomas and portal inflammation were occasional and inconspicuous in the untreated animals. Perl's positive iron deposition was intense in microgranulomas and in portal spaces of the three

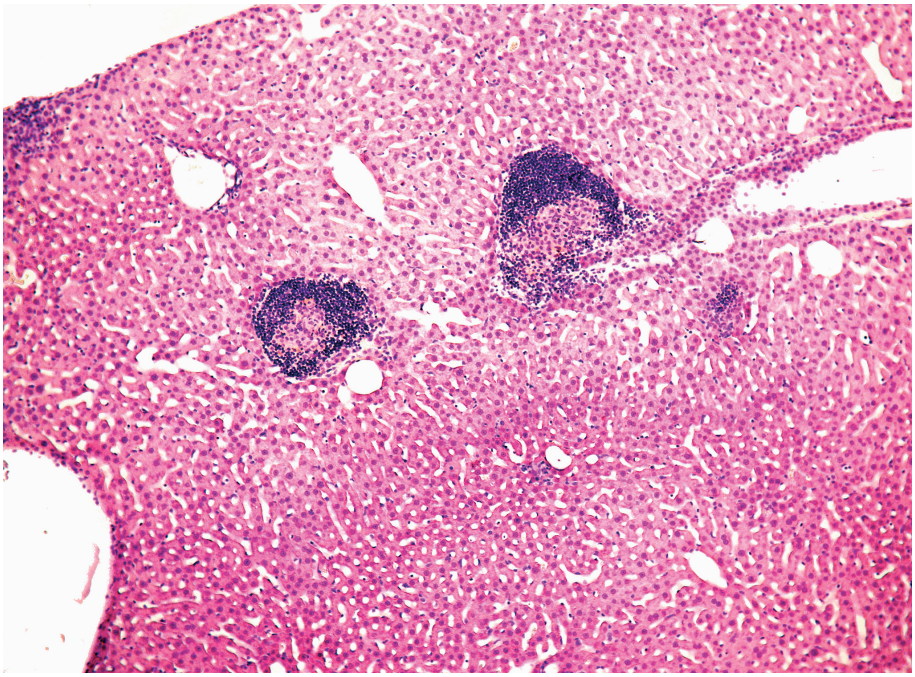

Fig. 3. Histological section of liver stained with hematoxylin and eosin in an experimental animal. Chronic inflammation with early non-caseificant granulomas were present in hepatic parenchyma. x 200

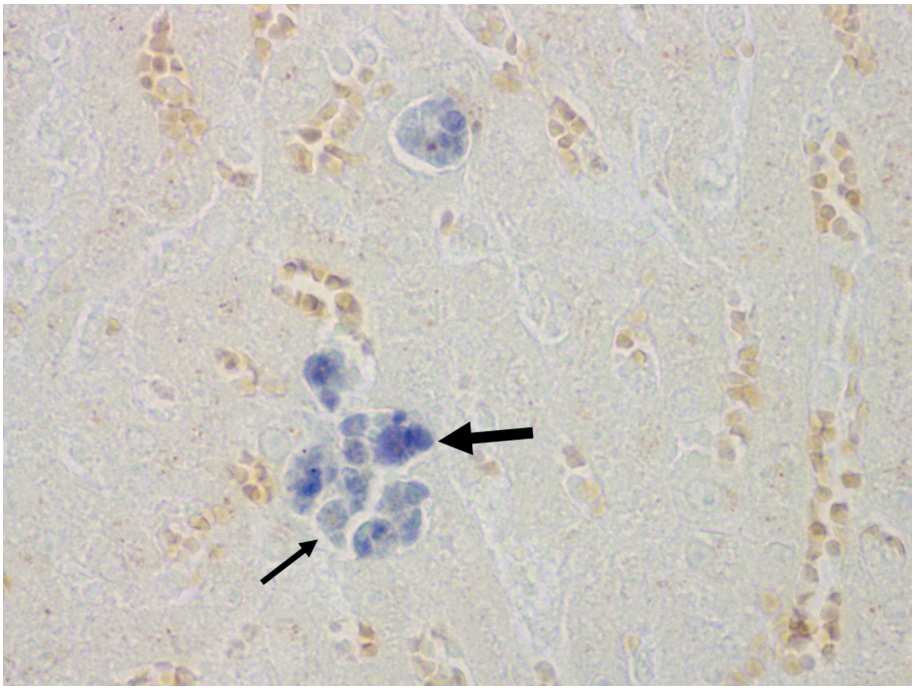

Fig. 4. Histological section of liver stained with solochrome azurine for Al detection in an experimental animal. Al inclusions appeared inside both degenerating hepatocyte (fine arrow) and Kupffer cell cytoplasms (thick arrow). x 670

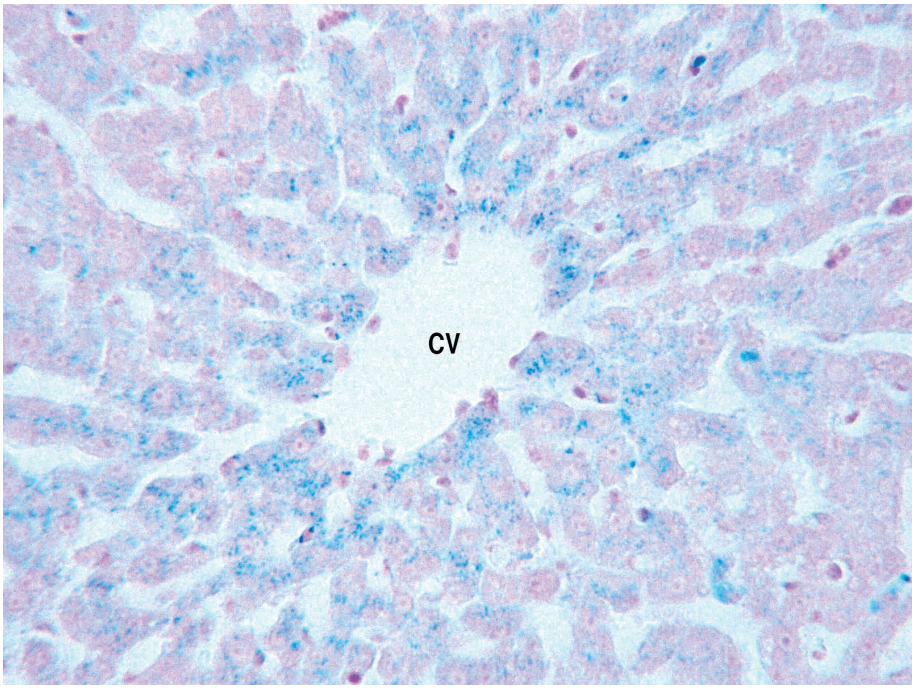

Fig. 5. Perl's staining in an experimental animal liver showing iron deposits inside hepatocyte cytoplasms, appearing in an acinar arrangement with a gradient towards the central vein (CV). x 200

groups of animals, but positivity in parenchymal cells appeared only in the Al-treated group. Iron-loaded hepatocytes showed multiple positive granules in their cytoplasms, the more abundant appearing closer to the central vein, following an acinar arrangement (Fig. 5).

Ultrastructurally, experimental animals showed mitochondria hyalinization, including small dilated cristae and/or fine dense granulation, disintegration of endoplasmic reticulum and clustering of ribosomes, which on many occasions appeared as aggregates around other organelles. Phagolysosomes were larger and more frequently encountered in hepatocytes (Fig. 6) and Kupffer cells (Fig. 7) of the experimental animals than in those of the control groups. Mitochondrion swellings often appeared in the experimental group, while they were less frequent in control animals.

Kidney

Two out of the six experimental animals showed normal histological features not distinguishable from the sham-injected and untreated animals. In the four remaining experimental animals, the same white deposits as in the liver were observed macroscopically on the surface of the kidneys. The capsule also appeared thickened and with inflammatory infiltrate in these latter animals, purulent perinephritis being severe in one of them, which also showed severe focal peritonitis (Fig. 8).

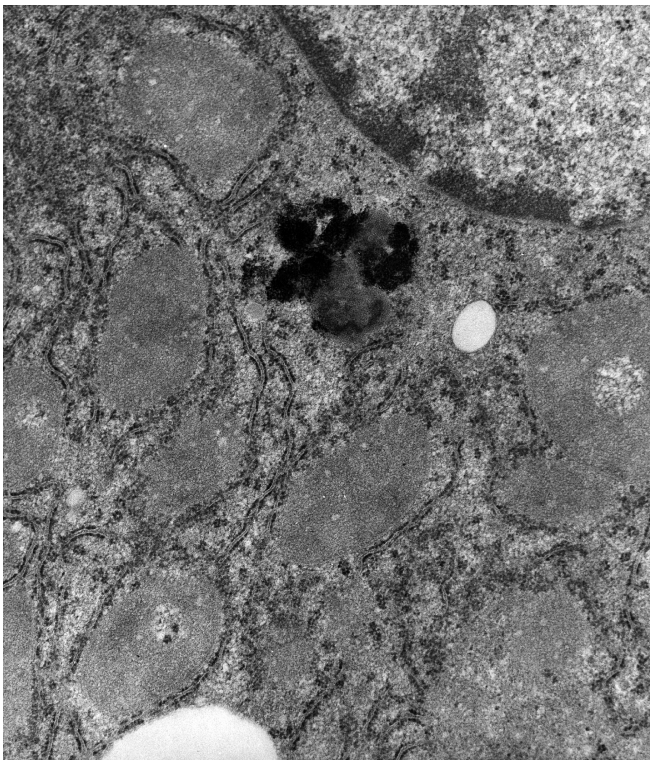

Fig. 6. Electronmicrograph of hepatocyte in an experimental animal. Mitochondrion hyalinization, clustering of ribosomes and large phagolysosomes could be seen. x 15,000.

A considerable accumulation of Al was noted by means of solochrome azurine stain filling the connective tissue surrounding the kidneys of the experimental animals. These deposits could also be observed as small greenish precipitates with hematoxilyn and eosin (Fig. 9). Distinct Perl's positive granules could be observed inside isolated mesangial cell cytoplasm in occasional glomeruli of the three groups of animals (Fig. 10). Nontheless, in one experimental animal, positive glomeruli were more frequently encountered and also a few tubular cells showed faint Perl's Prussian blue precipitate. In 2 out of the 6 experimental animals, tubular atrophy could be noted in the cortical region (Fig. 11).

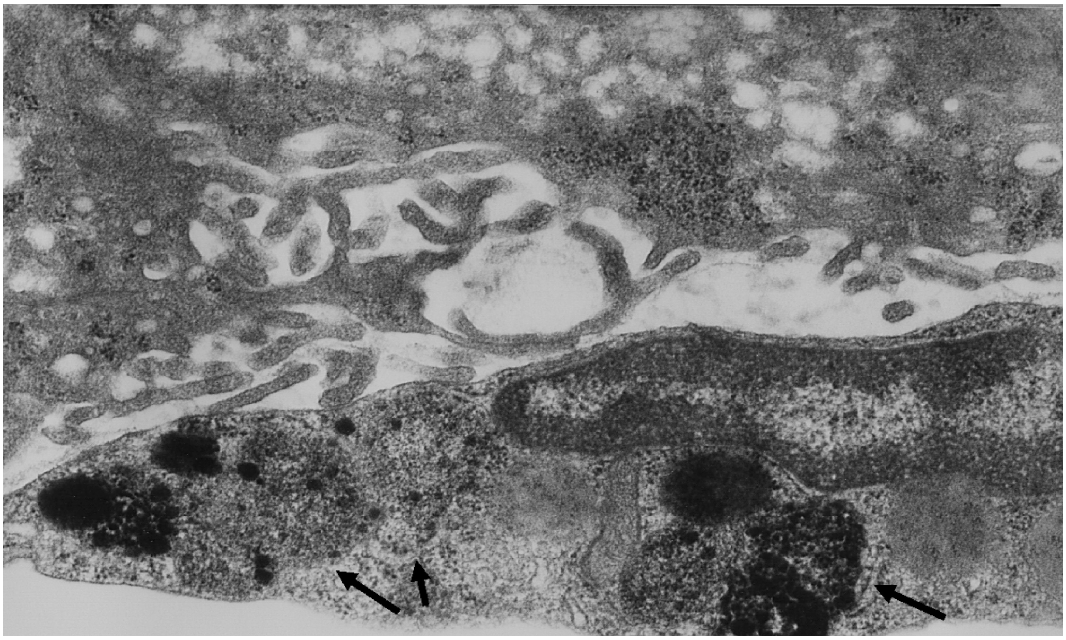

Fig. 7. Electronmicrograph of Kupffer cell in an experimental animal. Large phagolysosomes (arrows) stood out in the cytoplasm. x 12,000

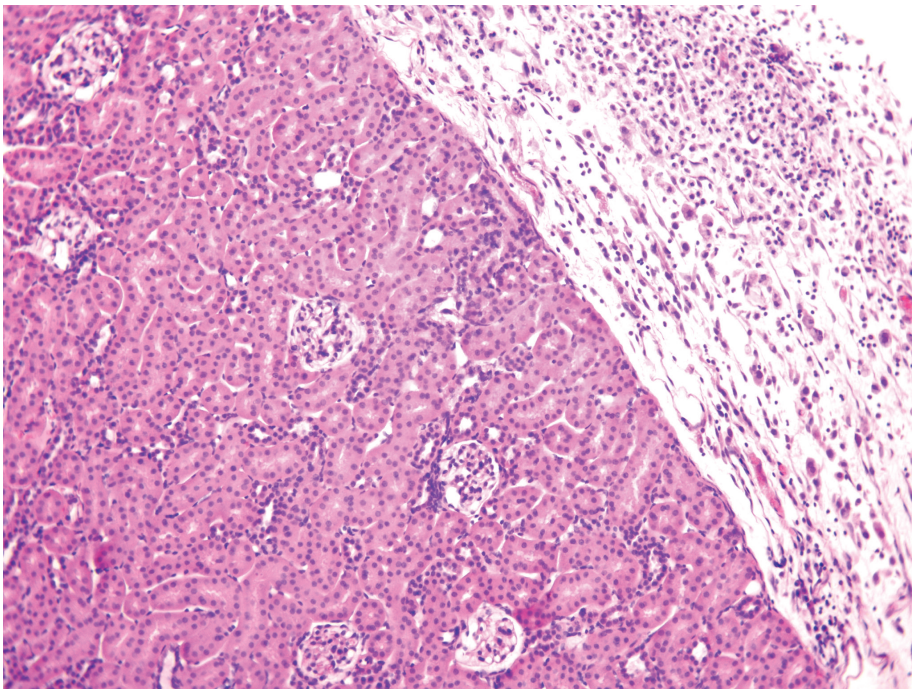

Fig. 8. Histological section of kidney stained with hematoxylin and eosin in an experimental animal. Suppurated perinephritis showing mono- and polymorphonuclear leukocytes could be seen surrounding the kidney. x 200

Ultrastructurally, no significant changes were found in the three different groups.

Discussion

The major histological change found in liver as a consequence of Al administration in this study was a chronic focal peritonitis. Adhesions of Glisson capsule to the diaphragm were also a common finding in the experimental animals. Adhesions to the diaphragm and intestine have been described by Fiejka et al. (1996) in mice injected with aluminum hydroxide via i.p., whereas

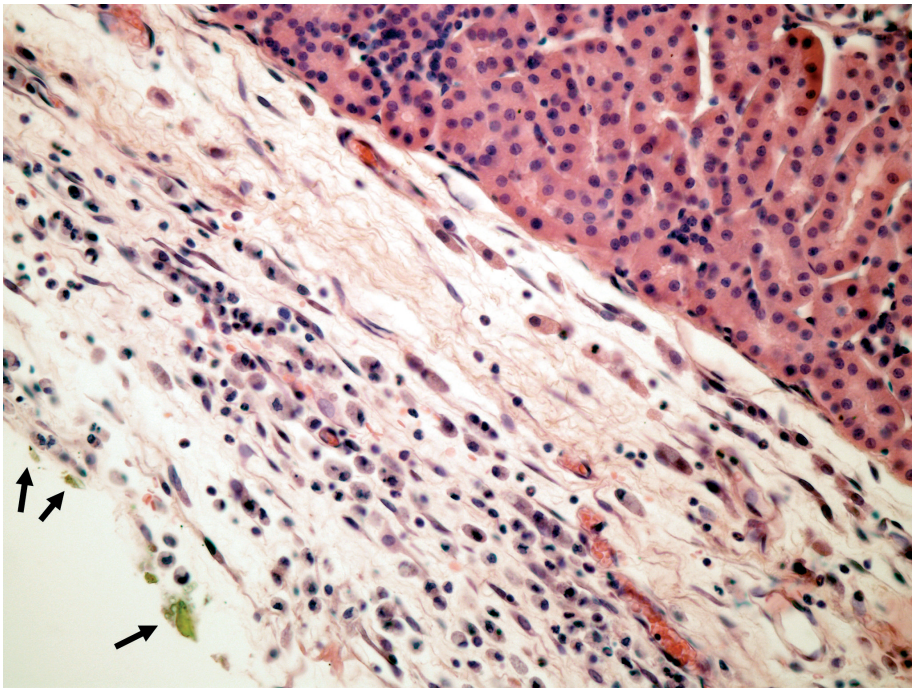

Fig. 9. Histological section of kidney stained with hematoxylin and eosin in an experimental animal. Acute inflammatory cells reappearing in a chronic perinephritis could be seen on the surface of the renal capsule, where Al deposits (arrows) were also present. x 480

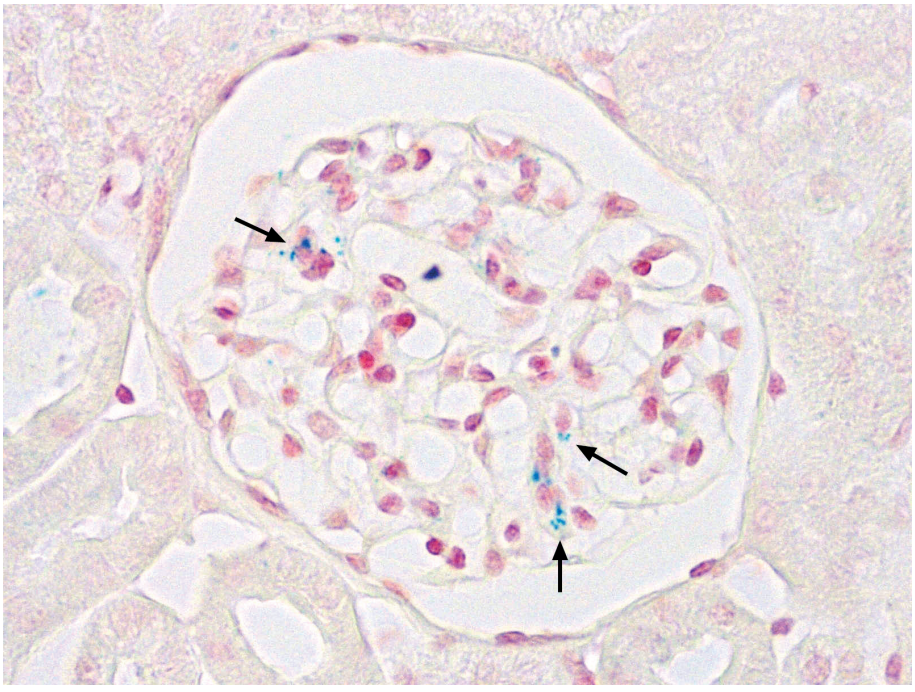

Fig. 10. Perl's staining in an experimental animal kidney showing iron deposits inside mesangial cell cytoplasms (arrows). x 400

Bertholf et al. (1989), using intra-venous Al maltol administration, did not report such adhesions, and Demircan et al. (1998), using an intraperitoneal canula, found no evidence of inflammation or irritation in the peritoneum nor adhesions. In our study, $AlCl_3$ provoked peritoneal irritation according to the previously-mentioned inflammatory response and the four clinically observed cases showing serum collection in the intraperitoneal space and hypersensitivity when injected. Irregularly scattered small foci of hepatocyte degeneration and necrosis with non-caseificant microgranulomas, together with mild portal inflammation including mononuclear cells, were also conspicuous in our experimental animals. The so-called alum-granulomas have also been found in mice injected with $Al(OH)_3$ (Fiejka et al., 1996), and rabbits (Bertholf et al., 1989), but no portal inflammation was reported in these studies. However, portal inflammation has been reported in rats treated with total parenteral nutrition or with $AlCl_3$ administered intraperitoneally (Demircan et al., 1998). Giant cells appeared close to granulomas, and have also been found in Al-intravenously-injected rabbits (Bertholf et al., 1989) and in Al-intraperitoneally-injected mice (Fiejka et al., 1996).

Interestingly, Ebina et al. (1984) used $AlCl_3$ injection as the control for Al-nitrilotriacetate (Al-NTA) treated rats. Whereas Al-NTA provoked coagulation necrosis, no remarkable change was found in livers from $AlCl_3$ injected animals. These findings are clearly opposed to ours and to other Al experimental studies where no coagulation necrosis has been described. On the other hand, the absence of histopathological changes in Ebina et al.'s $AlCl_3$ group could be interpreted on the basis of a shorter period of injection. In any case, the high variability of lesions described in the literature using different Al compounds suggests that their physical and chemical properties greatly determine Al toxicity and, therefore, particular properties of $AlCl_3$ solution, such as its lower pH, might account for the appearance of peritonitis, adherences and portal inflammation, rather than the intrinsic toxic effect of Al, without discarding eventual microbial contamination. Regarding granulomas, since they are also seen -to a lesser extent- in sham-injected animals, we must assume that although Al seems to increase their number, $AlCl_3$ injection is not the only factor responsible for alum-granuloma occurrence.

Al content is associated to elevations of iron in tissues (Ward et al., 2001; Ohtsuki et al., 2008). Our results show intense Perl's positive reaction in the granulomas and portal spaces of the three groups of animals, and in liver parenchyma only in the experimental group, in which a gradient towards the central vein is observed. Macrophages appear responsible for Perl's positivity in portal spaces, where Kupffer cells are normally larger and more phagocytically active (Bykov et al., 2004). This periportal positive reaction seems independent from Al administration since it is also present in the control groups. Regarding the centrilobular distribution of iron deposits in parenchymal cells, our results are in agreement with Stacchiotti et al. (2006) who described it after Al oral administration. This acinar arrangement of iron is maintained by the transferrin receptor pattern

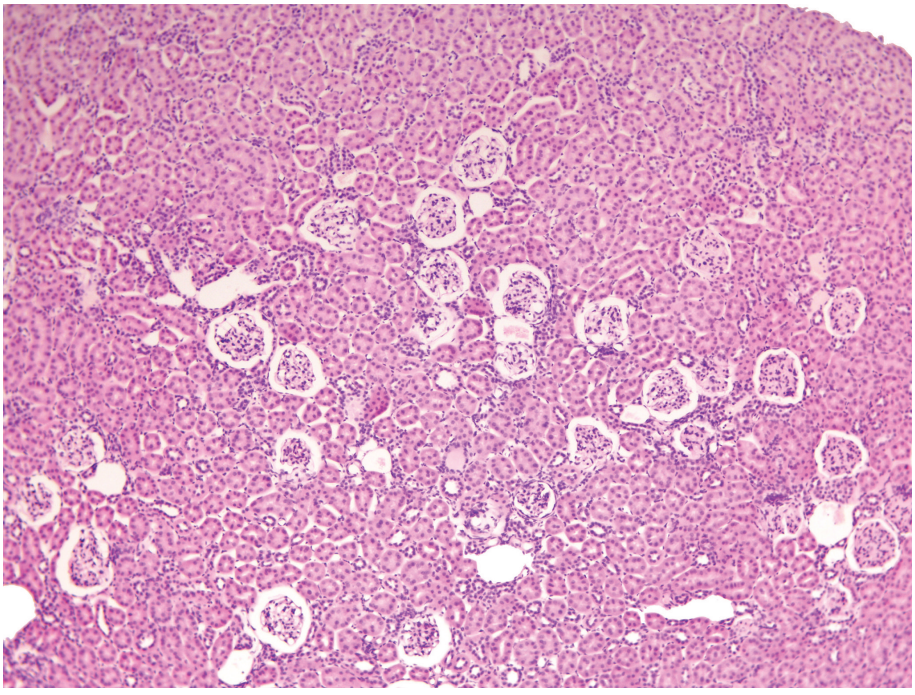

Fig. 11. Histological section of kidney stained with hematoxylin and eosin in an experimental animal. Tubular atrophy could be seen affecting convoluted tubules in the cortical region. x 120

(Stacchiotti et al., 2008). Al and iron share several physicochemical characteristics and interact competitively for transferrin receptors, and it has been shown that Al exposure affects transferrin- and non transferrin-dependent iron uptake (Pérez et al., 2005).

Although frequent in normal hepatocytes, phagolysosomes were more abundant and larger in animals treated with Al. Mitochondria changes specifically seen in experimental animals included hyalinization and cristae dilation. Other ultrastructural changes caused by Al in liver were disintegration of the endoplasmic reticulum and clustering of ribosomes in hepatocytes. Loss of ribosomes and rarefaction of the endoplasmic reticulum had been previously reported only in Kupffer cells after Al administration (Fiejka et al., 1996). The discrepancies in the literature regarding hepatocyte and Kupffer cell affection are also present in hemodyalized patients, since Al accumulation has been found in the lysosomes of both hepatocytes and Kupffer cells (Verbueken et al., 1984), whereas others reported that these changes spared Kupffer cells (Galle et al., 1987). Our data revealed that phagolysosomes were not only conspicuous in hepatocytes but that they could be even more frequently encountered in Kupffer cells. The role of dosage and Al species utilized might account for these differential effects, although this requires further investigation. Furthermore, no alterations were seen in liver after both intravenous and intramuscularly injected Al intoxication (Klein et al., 1988). Failure to find hepatic changes when the latter routes were used could be due to a more widespread distribution of Al in the body, especially in bone and spleen (Constantini et al., 1989) and to a slower absorption in the case of intramuscular injection. In any case, it seems clear that the liver undergoes a large influx of substances present in peritoneal space fluids via the portal vein; besides this, although the urinary tract is the main excretion route for Al, its biliary elimination also plays a considerable role. In this context, we found no cholestasis, contrasting with that reported in rats after i.p. administration of total parenteral nutrition (Demircan et al., 1998).

Regarding the kidneys, Bertholf et al. (1989) found accumulation of Al in the cortical renal tubules. In our study, accumulation of Al was restricted to the connective tissue surrounding the kidneys and, except for our two cases of mild cortical atrophy, we did not find any apparent kidney alterations; this also contrasts with the results of Bertholf et al. (1989), who reported acute proximal tubular necrosis and tubular atrophy in 50% of their Al-treated rabbits. These authors used a lower dose, albeit intravenously and during a much longer period (8-30 weeks). Likewise, major kidney damage, mainly in proximal tubules, has been reported after 6-month Al oral treatment in rats (Somova et al., 1997; Stacchiotti et al., 2006). Thus, our results could show the initial changes of the degenerative process because of our shorter exposure, but differences may also be due to their intravenous- or oral- instead of intraperitoneal-administration. In our study, the i.p. route would result in Al causing more toxicity in the liver, which agrees with the biochemical data by Ward et al. (2001) who, also using the i.p. route for Al administration, found iron deposition in the liver but not in the kidneys. Liver filters what directly comes from the intraperitoneal fluid via the porta vein and therefore hepatocytes are first to come into close contact with the possible toxicant present in the intraperitoneal space, whereas the kidneys suffer less aggression, which together with differential tissue susceptibility, would cause less alterations in these latter organs.

Iron deposition in the kidneys appears as an occasional feature in our study, which is in agreement with former biochemical (Ward et al., 2001) and histochemical (Stacchiotti et al., 2006) studies. Furthermore, the absence of significant iron deposition in renal parenchyma is consistent with the lack of Al accumulation in this organ after Al overloading. In addition to the reported occasional Perl's positive reaction in proximal tubules of Al-treated animals, a feature not previously described is our finding of isolated mesangial cells showing iron deposits both in experimental and in control groups.

As a final conclusion, we can consider that the intestinal barrier presents an important resistance to Al absorption which, together with renal excretion, means the Al body load is far from toxic levels. However, with deterioration of this intestinal barrier, an increase in absorption by acidification -as when ingested concurrently with citric or other acids-, when administered systemically or in renal failure, Al potential toxicity may arise, constituting a potent hepatotoxic element which causes, among other changes, focal peritonitis, mild portal inflammation and increase of phagolysosomes both in hepatocytes and Kupffer cells. These deleterious effects on the liver are greater when daily injections are administered in the form of $AlCl_3$ rather than $Al(OH)_3$ and intraperitoneally rather than intramuscularly, whereas oral administration does not seem to cause damage. Conversely, the toxic effect of $AlCl_3$ on kidney histology appears more severe after oral administration than when given intraperitoneally, although other factors such as differential animal species susceptibility and duration of treatment must also be taken into account.

Acknowledgements. We would like to thank Prof. Aleksandrowicz and Dr. Fiejka for their guidance and comments and Luis Santiago, Rogelio Martínez and Teresa Rodríguez for their technical assistance.

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Accepted January 24, 2011