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Acute effects of tetrabutylammonium chloride ionic liquid on the histological structure of liver and kidney in the mouse

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Abstract

In this paper, the acute toxicity of tetrabutylammonium chloride was approached through the 24 hours acute toxicity test, the major morphological changes induced in liver and kidney and the way in which this ionic liquid affects the catalase activity in mice. The results of our study show that average lethal concentration (LC50) in 24 h for the tetrabutylammonium chloride in mouse is 125 mg/Kg body weight. The histological observations proved that the acute exposure to the tetrabutylammonium chloride induces a series of histological changes both in liver and in kidney. The microscopic analysis of histological sections showed hypertrophies and hepatocyte vacuolations, glomerular compressions, thickening of the external layer of the Bowman'capsule, nephrocyte vacuolations as well as hypertrophies of the peri-tubular capillary network. The results of this study have also revealed a decrease in catalase activity, this process being more intense in individuals of the first two experimental groups.

Keywords: ionic liquids, mouse, toxicity, histopathology, liver, kidney, catalase

Introduction

Since their appearance, the ionic liquids have had a significant influence on organic chemistry, on biochemistry and "Green Chemistry" thanks to their unique physicochemical properties, manifested by their typical structure (Brennecke *& al*., 2001 [1]; Carda-Broch *& al.,* 2003 [2]; Gathergood *& al*., 2004 [3]). In recent years, the ionic liquids (ILs), thanks to their characteristic properties, not found to other substances, began to be used in chemical processes as new-generation solvents, replacing the conventional organic solvents which present considerable emissions of toxic fumes (VOCs). These properties refer to the negligible vapour pressure, high thermo-stability, high ionic and thermo-conductivity, fire-proof, melting points located, for the most of the ionic liquids, under the temperature of 100ºC and very good dissolvent properties, for both organic and inorganic compounds (Bernot & al., 2005 [4]). Although they have many industrial applications, the environmental transfer and the toxic potential of the ionic liquids are still largely unknown. Thanks to their negligible vapour pressure (cca. 10^{-11} Pa), it is believed that ionic liquids belong to the solvents category with "green character" and do not contribute to air pollution. But, they are water-soluble and can enter the environment through the underground water, presenting toxic potential to

aquatic ecosystems (Alfassi *& al*., 2003 [5]; Sheldon, 2005 [6]). Furthermore, thanks to the great stability of ionic liquids in water, these compounds can become perdurable pollutants in residual water. The toxic nature of ionic liquids determines the necessity to conduct certain additional eco-toxicological studies of ionic liquids on different species, in order to improve "the designing rules" in the synthesis of ionic liquids with low toxicity on integrated organisms into the environment. This is why, in recent years, testing the toxic potential of ionic liquids on different types of organisms and microorganisms are trialled in the whole world. A relatively small number of reports concerning the toxicological properties of ionic liquids have been presented so far. Thus, Pretti *& al.,* (2006) [7] tested the aquatic vertebrates' response to the administration of the 15 types of ionic liquids with different anions and cations. Bernot (2005) [4] studied the toxic potential of ionic liquids with imidazolic and pyridinic nucleus using different aquatic organisms as biological witness (*Physa acuta, Daphnia magna, Vibrio fisheri, Lemna minor, Pimephales promelas*). Couling *& al*., (2006) [8] establish the connection between the degree of toxicity of ionic liquids and the length of the alkyl chain attached to the imidazolic and pyridinic nuclei and to the quaternary ammonium cation of these salts, also showing that the toxicity of ionic liquids, to both the bacterium *Vibrio fischeri* and the crustacean *Daphnia magna*, gradually increases with the number of nitrogen atoms in the aromatic cation ring. Thus, the toxicity of ionic liquids increases from the ammonium ion > pyridinic nucleus > imidazolic nucleus > triazolic nucleus > tetrazolic nucleus. Cho *& al*., (2007) [9] evaluate the toxicity of ionic liquids with imidazolic nucleus (1-propyl-3-methylimidazolium bromide) [PMIM], 1-Butyl-3-methylimidazolium bromide [BMIM], 1-hexyl-3-methylimidazolium bromide [HMIM] and 1-octyl-3-methylimidazolium bromide [OMIM]) on the *Selenastrum capricornutum* freshwater algae. Li *& al.,* (2007) [10] studied the toxic effect of the 1-metyl-3-octylimidazolium bromide [C_8mim] [Br] on early embryonic development of the frog *Rana nigromaculata,* and later (2011, 2012) [11, 12] they studied the effect of the same ionic liquid on mouse and gold fish.

In this paper, the main objective was the assessment of the acute toxicity of tetrabutylammonium chloride on mouse, the establishment of the main histological changes produced by this ionic liquid in the liver and kidney tissue and how the catalase activity is affected, enzyme of the antioxidant system, involved in the reduction of H_2O_2.

Materials and methods

The acute toxicity of ionic liquid on mouse was evaluated by measuring its lethal effects after 24 hours of treatment and it was expressed as average lethal concentration (LC50). The test was carried out in accordance with the recommendations of Lorke (1983) [13] and Shetty Atkila (2007) [14]. For this purpose, five experimental groups of 5 mice/group were organised. Group treatment was an intraperitoneal injection with the following doses of tetrabutylammonium chloride: (1) 250 mg/Kg body weight (BW), (2) 125mg/Kg BW, (3) 62.5 mg/Kg BW, (4) 31.25 mg/Kg BW and (5) 15.62 mg/Kg BW. The average weight of mice in each group was 40 g. In order to establish the necessary dose of tetrabutylammonium chloride, serial dilutions were carried out in the 0.9% saline solution, which were reported to kilograms live weight. After determining the LC50, five groups of 7 mice/group were organized. Four of these groups were experimental, the animals from each group being injected intraperitoneally with tetrabutylammonium chloride, with the following doses: 125 mg /Kg BW – group 1, 62.5 mg/Kg BW – group 2, 31.25 mg/Kg BW - group 3, and 15.62 mg/Kg BW – group 4. The fifth group was the control group, which was injected with the same volume of 0.9% saline solution. In both cases, the volume of the injected solution was calculated according to body weight, not exceeding 1 ml/100 g body weight.

Organ sampling was conducted immediately after animals' death, and for groups with no mortalities, slaughter was done 10 hours after injection.

Biological material

The mice were originated from the Bucharest Cantacuzino Institute. The rearing conditions of the animals complied with the legislation in force, namely: mice were housed in plastic cages, in number of 5 mice/cage, on wood shavings litter, at 21 ± 1ºC air temperature, 55±5% air humidity, and a photoperiod of 12: 12 light-dark. All the mice had free access to the standard diet (pellets for rodents) and tap water. During the course of the experiment, mice were not fed.

Chemicals

Tetrabutylammonium chloride ($C_{16}H_{36}ClN$) was bought from Fluka Analytical (Switzerland). All reagents and stains for the histopathological study and for the determination of catalase were purchased from Merck (Germany).

Histopathological examination

For the histopathological examination, the samples taken from the liver and kidneys were fixed in 10% neutral formalin and included in paraffin blocks, after a prior dehydration in increasing alcohol solutions (70º, 80º, 90º, 100º) and clarification in two benzene baths. Paraffin blocks sectioning was carried out using the manually rotary microtome Leica, at a thickness of 4 μm. After adhering them on the microscope slides, the histological sections were stained with Mallory's trichrome method and were examined under Olympus CX41 research microscope fitted with digital camera and analysed using the QuickPHOTO Micro2.2 software for the histomorphometry.

The statistical analysis of the histomorphometry results was made using the Mann-Whitney U Test. The results are presented as the mean ± SE. Differences were considered to be statistically significant at $p<0.05$, distinct significant at $p<0.01$ and very significant at $p<0.001$.

Determination of catalase activity

Determination of the catalase activity was carried out using the photometric method proposed by Sinha (1972), on blood samples taken at the slaughter of animals through the caudal vena cava puncture. Results of this enzymatic test are presented in μMH_2O_2 Transf/min, ml, at 25ºC.

Results and discussions

LC50 of Tetrabutylammonium chloride ($C_{16}H_{36}ClN$) in mouse

The average LC50 lethal concentration of tetrabutylammonium chloride ($C_{16}H_{36}ClN$), injected IP to mice, was 125 mg/Kg BW, after 24 h.

Histopathological study of the liver

In the group that received 125 mg/Kg BW tetrabutylammonium chloride, the microscopic analysis of the histological sections of liver reveals hypertrophic processes manifested at the level of hepatocytes, sinusoidal capillaries, venules and centrolobular venae. The mono- or bi-nucleate hepatocytes present apparent nucleolus, and the cytoplasm is granular, in which microvacuoles presence is signalled (Figure 1a), with greater frequency in the exolobular and centrolobular segments of hepatic lobules. The average size of the hepatocytes is 23.7 μm in diameter; 88.5 μm in perimeter and area of 567.5 μm^2 (Table 1).

For individuals receiving 62.5 mg/Kg BW tetrabutylammonium chloride, the microscopic appearance of the histological sections from the liver was similar to that of the individuals belonging to the experimental group 1. Hepatocytes maintained their ordinate arrangement in bands converging towards the centrolobular vena. They have polygonal appearance, and their granular cytoplasm contains a smaller number of microvacuoles (Figure 1b). Also, they have a slightly hypertrophic appearance, with the average diameter of 23.1 µm, perimeter of 83.2 µm and the area of 507.1 μm^2 (Table 1). The hypertrophic processes are displayed at the venules, sinusoid capillaries and centrolobular venae, as well.

In experimental groups 3 and 4, whree animals were injected with 31.25 mg/Kg BW and 15.62 mg/Kg BW, respectively of tetrabutylammonium chloride, slight hepatocyte hypertrophies were reported (Figure 1c, 1d). Thus, in group 3 individuals the average diameter of hepatocytes was 21.5 µm, perimeter was 71.2 µm, and average value for area was 371.7 μm^2. In group 4 individuals these values were a little bit lower: average diameter 20.3 µm, average perimeter 70.8 µm, and average area 358.7 μm^2. The hepatocytes, arranged in ordinate bands, had intensively granular cytoplasm and spherical, central nucleus. Only on small areas vacuoles were present in the cytoplasm of these cells (Figure 1c, 1d).

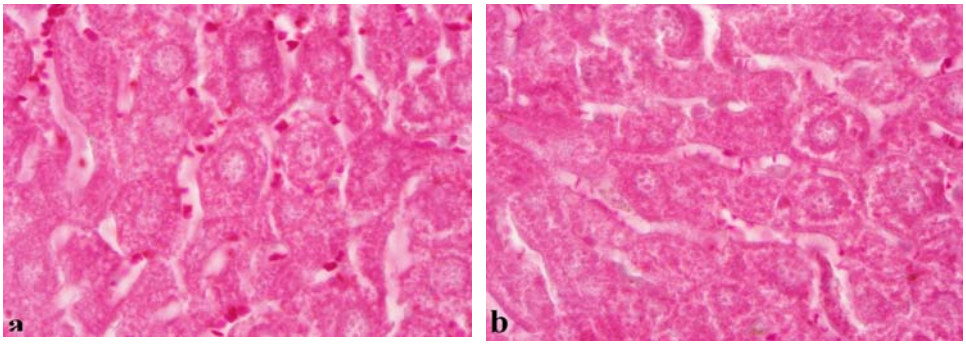
The histomorphometric analysis on liver sections in control individuals (group 5) reveals the smallest size hepatocytes (Figure 1e) compared to all experimental groups, as follows: 20,0 µm average diameter, 70,6 µm average perimeter and 364,2 μm^2 average area (Table 1). The hepatocytes cytoplasm presents very rare microvacuoles with a normal morphological appearance.

Table 1. The average size of the hepatocytes

Specification	Group 1	Group 2	Group 3	Group 4	Group 5 (control)
Diameter (µm)	23.7 ± 1.3^{xxx}	23.1 ± 2.8^{x}	21.5 ± 2.7	20.3 ± 2.1	20.0 ± 1.1
Perimeter (µm)	88.5 ± 4.2^{xxx}	83.2 ± 6.0^{xxx}	71.2 ± 4.6	70.8 ± 2.2	70.6 ± 4.6
Area (μm^2)	567.5 ± 52.5^{xxx}	507.1 ± 70.8^{xxx}	371.7 ± 47.9	358.7 ± 19.8	364.2 ± 45.8

$^{x}p<0.05$ compared with group 5 (control); $^{xxx}p<0.001$ compared with group 5 (control)

The statistical analysis of the histomorphometric data presented in Table 1 shows that the average value of the diameter, perimeter and area of the first two experimental groups differ statistically very significantly ($p<0.001$) from the values found in the control group, except for the hepatocyte diameter in individuals belonging to the experimental group 2, which differs statistically significant ($p < 0.05$) from the same parameter in control group.



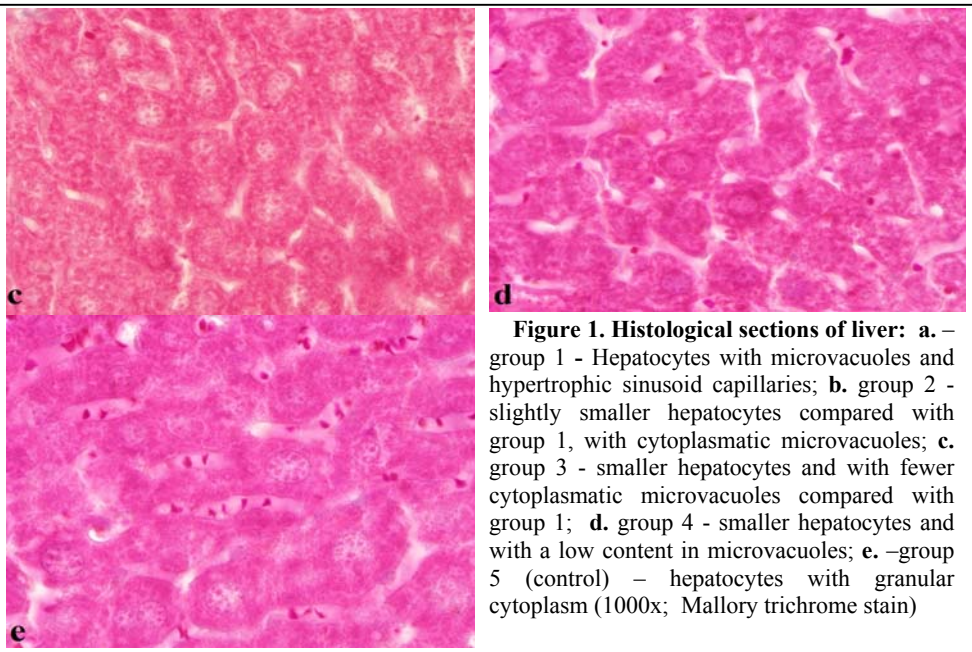


Figure 1. Histological sections of liver: a. – group 1 - Hepatocytes with microvacuoles and hypertrophic sinusoid capillaries; **b.** group 2 - slightly smaller hepatocytes compared with group 1, with cytoplasmatic microvacuoles; **c.** group 3 - smaller hepatocytes and with fewer cytoplasmatic microvacuoles compared with group 1; **d.** group 4 - smaller hepatocytes and with a low content in microvacuoles; **e.** –group 5 (control) – hepatocytes with granular cytoplasm (1000x; Mallory trichrome stain)

The histopathological study of the kidney

The microscopic analysis of sagittal sections of kidney reveals, in the case of individuals from group 1, a series of morphological changes at the renal parenchyma level. Thus, at the renal corpuscles level the glomerular capillaries are often ectasic. In the capsular area, on restricted territories, the presence of leukocyte infiltrates was signalled and at the level of Bowman capsule pericapsular fibrosis processes were present. The external layer of the Bowman capsule is thickening through the metaplasia of its pavimentous monostratified epithelium in a cubic or, on small areas, prismatic epithelium. The renal corpuscles appearance is heterogeneous (Fig. 2a), on extensive territories having compressed appearance, with a wide capsular space, and thickened external Bowman capsule (Fig. 2b). In the cortical peripheral area, under the renal capsule, the proximal and distal contort tubes have a disorganized appearance due to the numerous nephrocyte dystrophies. They are hypertrophying, their cytoplasm is heavily vacuolated, apical pole appears "ragged" and the cell membrane detaches from the subepithelial basal membrane. On vast areas of renal parenchyma, the uriniferous tubules are lined with cubic or prismatic monostratified epithelium. The nephrocytes have spherical, central nucleus, with evident nucleolus, and the cytoplasm is loaded with fine granulations and small vacuoles with uniform appearance (Fig. 2c). In the organ stroma, slightly fibrosed, the vascular network is observed, in particular the peritubular, hypertrophic capillaries, in the lumen of which blood cell elements are visible.

The sagittal sections through kidneys of the group 2 individuals reveal the same morphological aspects as in the case of the individuals from group 1, but of lower intensity. The renal corpuscles are heterogeneous in terms of size (Fig. 2d), from small corpuscles with compressed glomerulus and vast capsular space, to hypertrophic corpuscles, and the external layer of the Bowman capsule is thickening (Fig. 2e, 2f). The most abundant are the corpuscles with compressed glomerulus. The uriniferous tubules present cubic or prismatic monostratified epithelium, having microvilli at the apical pole. The nephrocytes' nucleus is spherical, central, and the intensely granular cytoplasm present reduced microvacuoles (Fig. 2f). Changes of the tubular epithelium appear in the subcapsular, cortical area. Thus, the nephrocytes are hypertrophying, theirs cytoplasms are clear. This appearance is the result of the macrovacuoles accumulation, and certain of them have the apical membrane broken. The

peritubular vascular network is hypertrophic and, on certain areas, congestive.

In the case of the individuals from experimental group 3, the microscopic analysis of sagittal sections through kidneys shows the overview of the two parts of renal parenchyma, cortical area and medular area, respectively, in whose stroma the hypertrophic peritubular capillary network is signalled and, on certain areas, with congestive appearance. The renal corpuscles are slightly heterogeneous as size. In comparison with the morphological aspects showed in the individuals of the first two groups, the small corpuscles, with compressed glomerulus and thickened external layer of Bowman capsule are rarer (Fig. 3a, 3b). The nephrocytes of the uriniferous tubules are slightly hypertrophic, with intensely granular cytoplasm (Fig. 3c), and at their apical pole, the brush border is evident. Among the granular cytoplasm nephrocytes, the hypertrophic, clear nephrocytes are also present, with cytoplasm loaded with large vacuoles. Their frequency is higher in the peripheral area of the cortical. Slight interstitial fibrosis processes are displayed on limited areas.

The microscopic analysis of the histological sections through the kidney of individuals from the experimental group 4 reveals the presence of renal corpuscles having similar sizes (Fig. 3d). The vascular glomerulus is slightly compressed, and the Bowman capsule external layer presents thickenings on more limited areas (Fig. 3e). The nephrocytes have granular cytoplasm (Fig. 3e), spherical, central nucleus, and there are extended microvilli at the apical pole. Among them, rare nephrocytes with vacuolar cytoplasm are present. The peritubular capillary network is slightly hypertrophic.

In the case of the individuals from the control group, the renal parenchyma does not show the morphological changes presented in the individuals from the experimental groups. However, thickenings of the Bowman capsule external layer, as well as slight glomerular compressions are visible (Fig. 3f, 3g). Also, the peritubular capillary network is no longer hypertrophic. The uriniferous tubules have a normal appearance, and hypertrophic nephrocytes, with vacuolated cytoplasm are present only on the cortical area periphery.

In all the experimental groups, the intraperitoneal administration of tetrabutylammonium chloride determines the hepatocytes hypertrophy and the occurrence of cytoplasmatic vacuoles, their frequency differing according to the administered ionic liquid dose. Thus, as it was presented in the histological figures, the cytoplasmatic vacuoles are much more frequent in the case of individuials from the experimental groups 1 and 2. Cytoplasmatic vacuolation phenomena were reported by Yu *& al.,* (2009) [15], in the mice hepatocytes, treated with 1-octyl-3-methylimidazolium bromide type ionic liquid. Pretti *& al.* (2006) [7] found such histological changes, induced by different types of ionic liquids, in the zebra fish (*Danio rerio*), at the level of skin and gill filaments. The histological changes induced by the ionic liquids, and especially by those with ammonium cation, correlate with their well-known surfactant action on the membranes, increasing the membrane permeability to the external ions, affecting the physical properties of the lipid bilayer (Pretti *& al.,* 2006, [7]). The toxic effect of the cationic surfactants suggests that the modifications of the cationic parts of ionic liquids are responsible for the toxic behaviour of these new solvents (Pretti *& al.,* 2006, 2009) [7, 16].

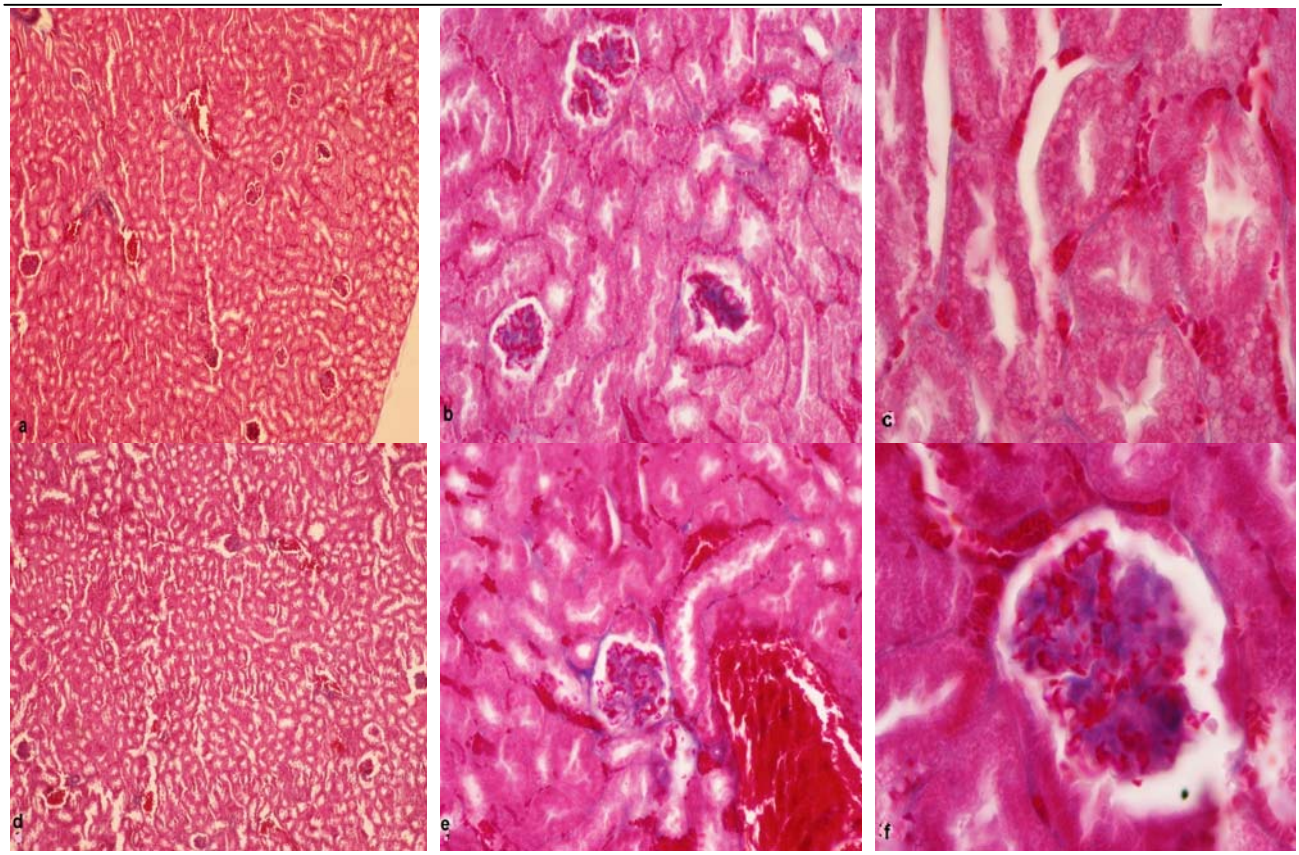


Figure 2. The histological appearance of the kidney: a, b, c. - group 1: overview – renal corpuscles of different sizes (a) (100x), heterogenous renal corpuscles, with thickening external layer of Bowman capsule (b) (400x) and the nephrocytes with vacuolar cytoplasm (c) (1000x); d, e, f. - group 2: overview – corpuscles of different sizes (d) (100x), Bowman capsule partially thickened (e) (400x), the epithelium metaplasia of the Bowman capsule external layer and nephrocytes with cytoplasmatic vacuoles (f) (1000x; Mallory trichrome stain)

The cytoplasmatic vacuoles are accumulated lipid droplets, suggesting a disruption of lipid metabolism, while the large cytoplasmic granulations are associated with the mitochondria hypertrophy, and with the entire cell, respectively. The liver is the main organ responsible for the metabolism of drugs and toxic chemical substances. Studies conducted by Franco *& al.,* (1986, 1991) [17, 18] suggested that the exposure to organic solvents can induce liver toxicity because most chemicals are metabolized in the liver and the generated toxic metabolites are the main cause of liver lesions. A large class of solvents, such as the cationic and amphiphilic solvents, has the ability to accumulate in the mitochondria, as a result of the mitochondrial membrane potential (Berson *& al.,* 1998, cited by Malaguarnera *& al.,* 2012 [19]). The accumulation of these solvents in the liver inhibits the β-oxidation of fatty acids into the mitochondria causing the appearance of lipid vacuoles and the transportation of electrons along respiratory chain (Berson *& al.,* 1998 cited by Malaguarnera *& al.,* 2012 [19]).

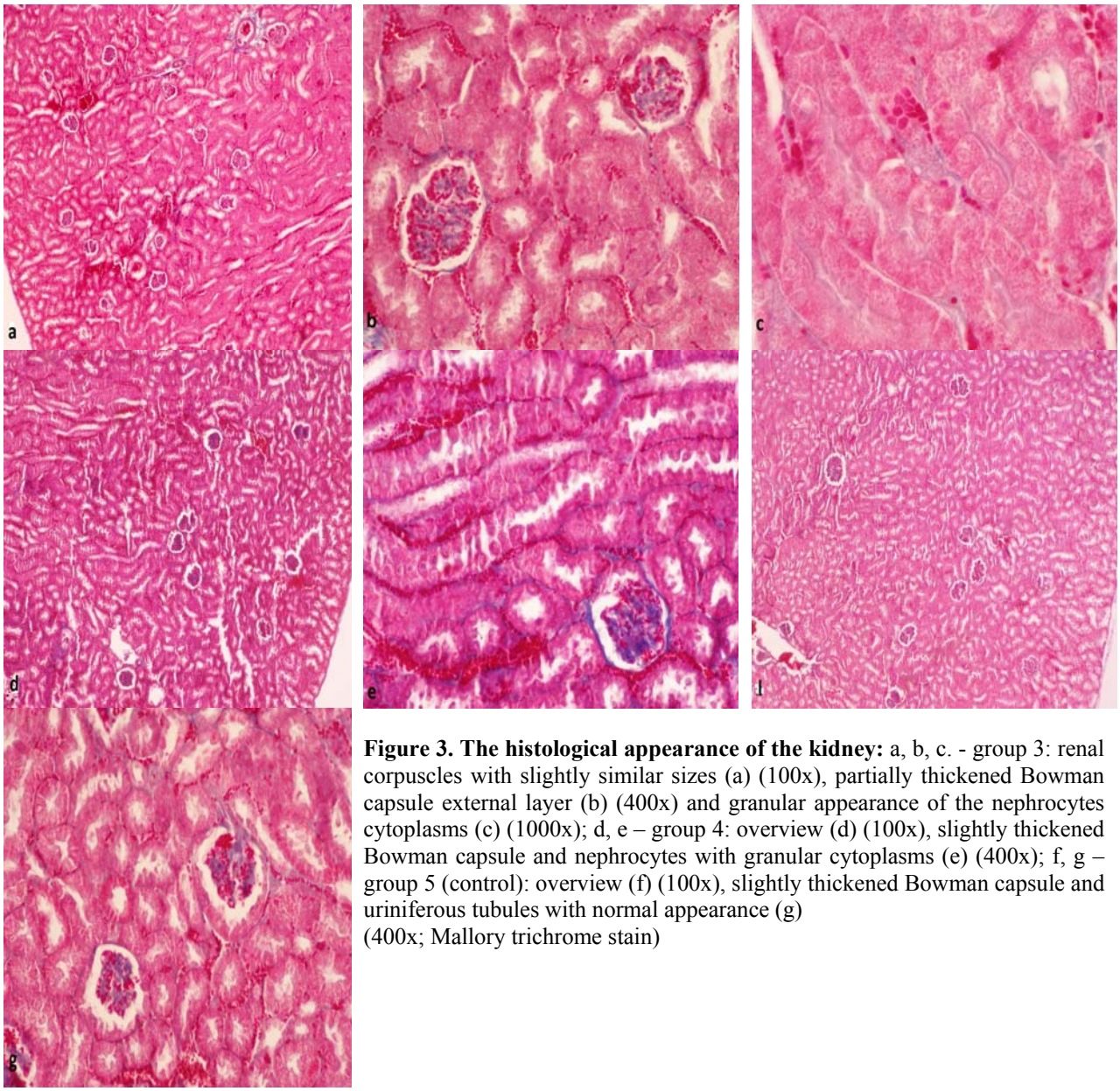


Figure 3. The histological appearance of the kidney: a, b, c. - group 3: renal corpuscles with slightly similar sizes (a) (100x), partially thickened Bowman capsule external layer (b) (400x) and granular appearance of the nephrocytes cytoplasms (c) (1000x); d, e – group 4: overview (d) (100x), slightly thickened Bowman capsule and nephrocytes with granular cytoplasms (e) (400x); f, g – group 5 (control): overview (f) (100x), slightly thickened Bowman capsule and uriniferous tubules with normal appearance (g)
(400x; Mallory trichrome stain)

The intraperitoneal administration of the tetrabutylammonium chloride induced, to all experimental groups, a series of morphological alterations at the renal parenchyma level, changes whose intensity depends on the ionic liquid concentration. Thus, in the case of the first two experimental groups, the renal corpuscles are heterogeneous in terms of size, with a high frequency of corpuscles with compressed vascular glomerulus, wide capsular space, and thickened epithelium of the Bowman capsule external layer. The uriniferous tubules nephrocytes are frequently hypertrophic, with vacuolar cytoplasm. Vacuolation aspects of the nephrocytes cytoplasm, together with intumescents of the uriniferous tubules, have also been reported by Li *& al.* (2011) [11], in gold fish, subjected to the acute toxicity of 1-methyl-3-ocylimidazolium bromide ionic liquid.

The catalase activity

The results of this study have revealed a higher decrease of the catalase activity in individuals belonging to the experimental groups 1 and 2 (Table 2), to which the injected amount of ionic liquid was 125 mg/Kg BW and 62,5 mg/Kg BW, respectively. To the other

groups, the obtained values were slightly lower compared to the control group that had the catalase value at 358.69 μMH_2O_2 Transf./min, ml, at 25°C (Table 2). The decrease of the catalase activity suggests the display of the oxidative stress, in particular mainly in the individuals of the first two experimental groups. This correlates with the morphological alterations that occur in the liver. Similar aspects, namely the decrease of the catalase activity, were reported by Yu *& al,* 2009 [15], which studied the effects of acute administration of 1-octyl-3-methylimidazolium bromide ionic liquid on the antioxidant system (superoxide dismutase, catalase, glutathione peroxidase and glutathione-S-transferase) of the mouse liver. Also, a series of recent studies reveal the involvement of ionic liquids in the inhibition of the enzymatic systems at the cellular level, mainly the acetylcholinesterase activity (Stock *& al.,* 2004 [20]; Jastorff *& al.,* 2005 [21]; Zhang *& al.,* 2005 [22]; Matzke *& al.,* 2007 [23]; Ranke *& al.,* 2007 [24]; Arning *& al.,* 2008 [25]; Stasiewicz *& al.,* 2008[26]; Torrecilla *& al.,* 2009 [27]).

Table 2. The values resulted for the catalase activity in the five groups

Specification	Group 1	Group 2	Group 3	Group 4	Group 5
Catalase (μMH_2O_2 Transf./min, ml, at 25°C)	**296**	**334**	**338**	**341.5**	**358.69**

Conclusions

The acute toxicity test result at 24 hours proved that in mouse LC50 for the tetrabutylammonium chloride was 125 mg/Kg BW, and the mice exposure to this ionic liquid induced a series of histopathological alterations in liver and kidney. Thus, the histological examination of the sections of liver and kidney indicated hepatic vacuolisations with cellular hypertrophies, epithelial metaplasia of the Bowman capsule external layer, glomerular compressions, as well as nephrocyte cytoplasmatic vacuolisations. The intensity of these changes is different, depending on the ionic liquid concentration. Also, this study shows that the acute administration of the tetrabutylammonium chloride ionic liquid determines a decrease of the catalase activity, indicating the oxidative stress presence.

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