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Studies on fate and toxicity of nanoalumina in male albino rats: some haematological, biochemical and histological aspects

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Abstract

The work aimed to evaluate the nanoalumina toxicity on the histological architecture, some haematological and biochemical aspects in male albino rats, during acute and sublethal experiments. Rats, in acute experiments, were injected with a single-acute dose of 3.9 g or 6.4 g or 8.5 g of aluminium oxide (Al_2O_3) kg^{-1}, whereas those of sublethal were injected with 1.3 g of Al_2O_3 kg^{-1} 2 $days^{-1}$. One-way analysis of variance indicated that injected doses and the experimental periods were significantly affected by haemoglobin (Hb) content; haematocrit value (Hct); white blood cell (WBC) count; blood platelet (Plt) count; mean corpuscular volume (MCV); mean corpuscular Hb (MCH) and MCH concentration (MCHC). In acute experiments, Hct, WBC count, MCV and Plt were significantly higher than the corresponding controls, whereas Hb, MCH and MCHC markedly decreased. In comparison with the related controls after 1, 3 and 7 days post-injection, red blood cell count, Hb, Hct, WBC count, Plt and MCV were significantly increased, but begun to decrease after 14 or/and 28 days and were associated with a marked decrease in MCH and MCHC. In serum of rats injected with acute or sublethal dose, the concentrations of total protein (TP) and total lipid (TL) were significantly lesser than the corresponding controls, whereas the levels of urea, uric acid, creatinine and the activities of aspartate aminotransferase and alanine aminotransferase were markedly increased. The injected doses were directly proportional with all the studied biochemical parameter, except the TL and TP that exhibited a negative correlation. Histologically, the highest acute and sublethal doses of nanoalumina caused hepatic irregular disarray, necrosis to the hepatic and Kupffer cells that are associated with congested blood sinusoids. The renal tissues characterized by the appearance of inter-tubular congestion that is accompanied by the dilation of the vascular glomeruli that completely occupied Bowman's capsule and accompanied with partial disappearance of the renal tubule's brush border. The brain showed a progressive degeneration of neurons in both the experiments.

Keywords

Nanoalumina, rats, acute, sublethal, haematological, biochemical, histological

Introduction

Nanoparticles are classified as being materials in which at least one dimension of the material is less than 100 nanometers in diameter (Burklew et al., 2012). The study of nanoparticles is becoming an area of research interest due to their unique properties, such as having increased electrical conductivity, ductility, toughness and formability of ceramics, increasing the hardness and strength of metals and alloys and by increasing the luminescent efficiency of semiconductors (Rittner and Abraham, 1998).

Nanoparticles are used in an industrial setting because they can be used to manufacture lightweight, strong materials as well as act as pigments in products such as paints, sunscreens and cosmetics (Burklew

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et al., 2012). Because nanoparticles have a large surface area to volume ratio, the use of nanoparticles in both industry and daily life is greatly increasing in realms that include advancing the quality of everyday materials and processes, improving the function of electronics and information technology, allowing more sustainable energy applications and acting as key players in environmental remediation applications (Ali, 2013). Humans are exposed to nanoparticles via several possible routes, including inhalation, dermal absorption and gastrointestinal tract absorption. Due to their unique properties such as small size and corresponding large specific surface area, nanomaterials may impose different biological effects from their micro-scale material counterparts (Nel et al., 2006). However, to date, toxicological and environmental effects of nanomaterials remain largely unknown.

Aluminium (Al) has been proposed as an environmental factor that may contribute to some diseases, affect several enzymes and other biomolecules and induced free radical-mediated cytotoxicity (Yousef et al., 2007). Combined daily administration of Al (260 mg kg^{-1}, oral) and ethanol (2 g kg^{-1}, oral) to male rats for 30 days was found to significantly decrease food intake, body weight gain and serum total protein (TP) content (Rajasekaran, 2000). Al could reduce the activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, acid phosphatase and phosphorylase in liver and testes (El-Demerdash, 2004). A long-term exposure to Al could impair spatial learning abilities and increases anxiety in rats (Sethi et al., 2008). Winklhofer et al. (2000) reported that rats with iron deficiency had a larger intestinal uptake and higher concentrations of Al in liver, spleen and plasma than the control group, whereas iron overload decreased intestinal absorption and tissue concentrations of Al.

Blood or haematological parameters are probably the most rapid and detectable variables in the detection of stress and assessing different health conditions (Uboh et al., 2012). In clinical and experimental studies, the significance of blood parameters cannot be overemphasized (Uboh et al., 2010). Several literature reports indicated that haematological profile of different animal species may be influenced adversely by different chemicals. Transient changes are reported in the haematology rainbow trout, *Oncorhynchus mykiss*, under the effect of copper nanoparticles (Shaw et al., 2012). Both content of haemoglobin (Hb) and value of haematocrit (Hct) of rats reduced significantly after intra-peritoneal injection with aluminium sulphate as compared to controls (Farina et al., 2002). The stainless steel implantation caused a slight increase in Hb, Hct and red blood cell (RBC) count associated with a significant decrease in total white blood cell (WBC) count of male Wistar rats (Fadl-allah et al., 2011). Therefore, assessment of haematological and biochemical parameters are highly useful and benefit as markers for the extent of the deleterious effect of foreign substances on the blood constituents of an animal.

The present work is the third part of our series to evaluate the toxicity of nanoalumina (aluminium oxide nanoparticles (Al_2O_3-NPs)) on the biological system. The study was designed to assess the expected effects of nanoalumina on some haematological and biochemical parameters and the possible changes in the histological architecture of the brain, hepatic and renal tissues, during the acute and sublethal experiment.

Material and methods

Experimental animal

Healthy adult male albino rats, weighing 115 $\pm$ 5 g, were used as the mammalian experimental model. Rats were purchased from the animal house of the National Research Center (NRC), Giza, Egypt. Rats were acclimatized to the laboratory conditions for 2 weeks prior to experiments and housed in polyethylene cages in air conditioned animal house (at a temperature of 23 $\pm$ 1°C, relative humidity of 20.37% and cyclic day light for 12 h day^{-1}) and access to water and balanced diet *ad libitum*. The food debris and faeces were removed daily in order to keep sawdust dry throughout the experiments. All the experimental procedures were conducted in accordance with the general international guideline principles on the use of living laboratory animals in scientific research (Council of European Communities, 1986) and were approved by the Ethical Committee of Cairo University, Faculty of Sciences, Cairo, Egypt.

Chemicals

Nanoalumina (Al_2O_3-NPs), with an average diameter of 13.6 $\pm$ 3.5 nm, were purchased from Sigma-Aldrich (Ward Hill, Massachusetts, USA; 99.98% purity, Product number 718475, CAS number 1344-28-1, pH 9.4–10.1, boiling point 2.980°C, melting point 2.040°C and density 4.0 g cm^{-3}). Nitric acid, with a purity of 99%, was purchased from Chemical Company of El-Gomhouria (Cairo, Egypt). The Al_2O_3-NPs are ultrasonicated in deionized water

Table 1. Design for acute and sublethal study to clarify the experimental conditions throughout the course of experiments.

	Acute experimental groups				Sublethal experimental groups	
Experimental conditions	I	II	III	IV	V	VI
Saline	+++	−−−	−−−	−−−	+++	−−−
Al_2O_3-NPs (g kg^{-1} bw)	−−−	3.9[a]	6.4[b]	8.5[c]	−−−	1.3[d]
Dosing	Single				Day after day	
Sample size/group	5 rats				25 rats	
Sampling	After 2 days				After 1, 3, 7, 14, 28 days	

LD_{50}: median lethal dose; Al_2O_3-NP: aluminium oxide nanoparticles.
[a]Equivalent to 30% of LD_{50} at 48 h.
[b]Equivalent to 50% of LD_{50} at 48 h.
[c]Equivalent to 65% of LD_{50} at 48 h.
[d]Equivalent to 10% of LD_{50} at 48 h.
+++: Indicated that rats were injected with saline (group V) or nanoalumina (group VI).
−−−: Indicated that rats were not injected with saline or nanoalumina.

using the biologics ultrasonic homogenizer (Model 150VT) immediately before the administration, following the vibration at 20 kHz, with continuous pulse of 0.4 resulting in a power output of 40 W, for 5 min pre-administration. The average diameter of Al_2O_3-NPs, after ultrasonication, was found to be 9.38 $\pm$ 1.6 nm. The characterization of non- and ultrasonicated Al_2O_3-NPs (shape, diameter and aggregation) was estimated by the negative stain transmission electron microscope technique, according to the method described by Balasubramanyam et al. (2009).

Experimental design

The present study was designed to estimate the effects of intraperitoneal injection of ultrasonicated Al_2O_3-NPs on some haematological, biochemical and histological changes during acute and sublethal experiments. As estimated by Ali (2013), the median lethal dose (LD_{50}) of Al_2O_3-NPs at 48 h was 12.88 g kg^{-1}.

For acute experiments, 20 rats were divided randomly to 4 groups with 5 rats in each. The experimental rats of the first group were intraperitoneally injected with saline (group I, control), whereas the second to the fourth group were injected with a single acute dose of 3.9 g (30% of LD_{50}) or 6.4 g (50% of LD_{50}) or 8.5 g kg^{-1} (65% of LD_{50}), respectively. For sublethal experiments, 50 rats were divided randomly into 2 main groups, that is, control (group V) and Al_2O_3-NP-injected rats (group VI), each with 25 rats. Rats of group V were intraperitoneally injected with saline, whereas those of group VI were injected, day after day, with a sublethal dose of 1.3 g kg^{-1} body weight (bw) 2 $days^{-1}$, (1/10 of LD_{50}), over a period of 28 days. The design of the acute and sublethal experiments were summarized in Table 1.

Sampling

Rats were fasted 12 h prior to the sampling with free access to water. After 2 days (for acute experiments) and 1, 3, 7, 14 and 28 days (for sublethal experiments), the experimental animals were anaesthetized and killed by decapitation and the blood was collected in two parts. The first part was collected in a dry clean tube with ethylenediaminetetraacetic acid as an anticoagulant for the estimation of RBC count, Hb content, Hct value, WBC count, blood indices (mean corpuscular volume (MCV); mean corpuscular Hb (MCH); MCH concentration (MCHC) and blood platelet (Plt) count). The second part of blood collected in test tubes, without anticoagulant, left at the laboratory conditions for 30 min, and then centrifuged at 3000 r min^{-1} for 10 min. The supernatant serum was directly pulled by Pasteur pipette and stored at −20°C for biochemical assay. After the collection of blood, rats were dissected to obtain the brain, liver and kidneys that were fixed in Bouin's fixative to prepare the tissues for the histological examination.

Haematologically, the RBC count, Hb content, Hct value, WBC count, blood indices (MCV, MCH and MCHC) and blood Plt were measured by HA-Vet CLINDIAG (B-1177-78, G.D. Colony Mayur Vihar Phase-III, Delhi-110096, India) system blood auto-analyzer.

Biochemically, the concentrations of TPs, uric acid, urea, total lipids (TLs) and activities of transaminases (AST and ALT) were determined. The concentrations

of TP (in grams per decilitre) were estimated by Biomed (83 Abdel-Hamid Badaway St., Heliopolis, Cairo, Egypt) according to the method described by Yatzidis (1987). The TLs, uric acid, urea and creatinine (all measured in milligrams per decilitre) serum contents were determined by Bio-diagnostic (29 tahreer St., Dokki, Giza, Egypt) according to the methods described by Zollner and Kirsch (1962), Barham and Trinder (1972), Fawcett and Soctt (1960) and Tietz (1986), respectively. The activities of AST and ALT were estimated colorimetrically by Diamond (23 El-Montzh St., Heliopolis, Cairo, Egypt) according to the method described by Young (2001). The activities of AST and ALT were expressed as a unit activity of enzyme per litre of blood ($U\ L^{-1}$).

Histological investigations

The brain, liver and kidney of controls rats and those of rats injected with ultrasonicated Al_2O_3-NPs were fixed for 24 h in Bouin's fixative and then washed with 70% ethyl alcohol. The manual routine histological technique was used for sectioning the tissues at 10 μm as described by Drury et al. (1967), and then the samples were dehydrated in ascending concentrations of ethanol (70, 80, 90, 95 and 100%). The sections were then stained with haematoxylin and eosin dyes and mounted by Canada balsam and examined by light microscope.

Statistical analysis

The present data were analyzed by IBM SPSS Statistics (Statistical Package for the Social Sciences, SPSS version 20). Two-way analysis of variance (ANOVA) was applied to test the effects of injected doses, experimental periods and their interactions on the studied parameters, during the sublethal experiments, whereas one-way ANOVA was used to test the effects of acute doses of Al_2O_3-NPs. The ANOVA *post hoc* (least significant difference (LSD)) was used to compare between various groups. Regression analyses and correlation coefficient were applied to fit the relationships between the different studied variables. Data were represented as mean ± SEM.

Results

Haematological parameters

The RBC count, Hb content, Hct, WBC count, the blood Plt, MCV, MCH and MCHC of the adult male albino rats were estimated during the acute and sublethal experiments.

In acute experiments, one-way ANOVA revealed that the administered doses of Al_2O_3-NPs (0, 3.9, 6.4 and 8.5 $g\ kg^{-1}$ bw) had significant effects on all studied haematological parameters except RBC count (Table 2). According to the LSDs, at all acute doses, the WBC count, MCV and blood Plt count were significantly higher than the corresponding controls, but the MCHC was markedly depleted whereas the RBC count increased insignificantly (Table 2). The Hb contents and MCH, of groups III and IV, showed significant decrease in comparison with the corresponding controls, but the Hct values increased (Table 2). The regression analysis indicated that the injected acute doses of Al_2O_3-NPs exhibit inverse relationship with Hb contents, MCH and MCHC, whereas Hct values, MCV, WBC count and blood Plt showed a direct relationship with correlation coefficient (r) of −0.62, −0.72, −0.88, +0.78, +0.87, +0.92 and +0.92, respectively (Table 2).

In sublethal experiments, two-ways ANOVA indicated that the experimental time (1, 3, 7, 14 and 28 days), injected doses (0 and 1.3 g) and their interaction were significantly affected the RBC count, Hb contents, Hct values, WBC count, MCV, MCH and MCHC, whereas the blood Plt count was markedly influenced by the injected doses only (Table 3). At all the experimental periods, the LSD indicated that the RBC count and Hb contents were significantly higher than the corresponding controls, but the Hb contents were depleted markedly after 14 and 28 days post-injection (Table 4). After 1 and 3 days of Al_2O_3-NPs injection, the Hct value and WBC count were significantly increased in comparison with the corresponding controls, but after 7, 14 and 28 days, the leucocyte count was insignificantly lower than the corresponding controls (Table 4). After 1 day of injection with nanoalumina, the MCV was significantly higher than the corresponding controls, while after 14 and 28 days, the MCH and MCHC were markedly decreased and became significantly less than the corresponding controls (Table 4). In rats of group VI, the experimental periods showed direct correlation with the RBC count, Hb contents, Hct value, WBC count, MCV, MCH and MCHC, whereas the blood Plt exhibited positive relationships with correlation coefficients (r) of −0.74, −0.76, −0.75, −0.76, −0.93, −0.69, −0.89 and +0.48, respectively (Table 4). These relationships indicated that all the haematological parameters were influenced by the injected sublethal dose ($1.3\ g\ kg^{-1}$) throughout the experimental periods.

Table 2. The RBC count (10^{12} L^{-1}), Hb (g dL^{-1}), Hct (%), MCV (*fl*), MCH (ρg), MCHC (g dL^{-1}), WBC count (10^{9} L^{-1}) and the blood Plt count (10^{9} L^{-1}) of control male albino rats (group I) and those injected with a single acute dose of Al_2O_3-NPs (3.9 g or 6.4 g or 8.5 g kg^{-1} bw) after 2 days of injection.[a]

Experimental groups	RBC count (10^{12} L^{-1})	Hb (g dL^{-1})	Hct (%)	MCV (*fl*)	MCH (ρg)	MCHC (g dL^{-1})	WBC count (10^{9} L^{-1})	Plt count (10^{9} L^{-1})
Group I (control)	6.34 ± 0.40	12.65 ± 0.31	31.90 ± 1.18	48.74 ± 1.23	19.24 ± 0.12	39.44 ± 0.87	3.66 ± 0.44	262.60 ± 15.7
Group II (0.39 mg g^{-1}) (% Change)[d]	6.56 ± 0.24 (+3.47%)	12.56 ± 0.45 (−0.71%)	33.62 ± 1.22 (+5.39%)	53.72 ± 1.42[b] (+10.22%)	19.20 ± 0.13 (−0.21%)	34.14 ± 0.48[c] (−13.44%)	5.03 ± 0.17[b] (+37%)	336.64 ± 7.3[b] (+28.19%)
Group III (0.64 mg g^{-1}) (% Change)[d]	6.59 ± 0.15 (+3.94%)	11.43 ± 0.18[b] (−9.64%)	37.87 ± 0.52[e] (+18.71%)	55.37 ± 0.42[c] (+13.60%)	18.10 ± 0.23[e] (−5.92%)	32.82 ± 0.26[c] (−16.78%)	8.04 ± 0.40[c] (+120%)	721.60 ± 41.6[c] (+174.79%)
Group IV (0.85 mg g^{-1}) (% Change)[d]	6.84 ± 0.04 (+7.89%)	11.17 ± 0.43[b] (−11.69%)	38.58 ± 0.98[c] (+20.94%)	58.50 ± 0.13[c] (+20.02%)	17.93 ± 0.35[e] (−6.81%)	32.31 ± 0.56[c] (−18.08%)	11.78 ± 0.25[c] (+222%)	942.75 ± 15.3[c] (+259%)
Effect of doses (One−way ANOVA) (*p* value)	$F_{3,16} = 0.068$, (>0.05)	$F_{3,16} = 4.47$, (<0.05)	$F_{3,16} = 10.3$, (<0.01)	$F_{3,16} = 17.785$ (<0.0001)	$F_{3,16} = 9.431$ (<0.01)	$F_{3,16} = 31.15$ (<0.0001)	$F_{3,16} = 115.5$, (<0.0001)	$F_{3,16} = 10.3$, (<0.01)
Fitting equation[f]	$y = -0.11x+13.0$	$y = 1.09x+29.8$	$y = 1.46x$-0.86	$y = 1.03x+49.4$	$y = -0.28x+20.2$	$y = -0.40x+35.6$	$y = 3.38e^{0.138x}$	$y = 1.46x$-0.86
Correlation coefficient	+0.32	−0.62	+0.78	+0.87	−0.72	−0.88	+0.92	+0.92

RBC: red blood cell; Hb: haemoglobin; Hct: haematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular Hb; MCHC: MCH concentration; WBC: white blood cell; Plt: platelet; ANOVA: analysis of variance; Al_2O_3-NP: aluminium oxide nanoparticles.

[a]Each value is a mean of five rats ± SEM.

[b]Significant difference in comparison with the corresponding controls at $\alpha = 0.05$.

[c]Significant difference in comparison with the corresponding controls at $\alpha = 0.0001$.

[d]Percentage of changes in comparison with the corresponding controls.

[e]Significant difference in comparison with the corresponding controls at $\alpha = 0.01$.

[f]The fitting equation that confirmed the relationship and correlation coefficients of the administered-doses of nanoalumina with the studied haematological parameters. $p > 0.05$: insignificant effect; $p < 0.05$, $p < 0.01$ and $p < 0.0001$: significant effects at $\alpha = 0.05$, 0.01 and 0.0001, respectively.

Table 3. Two-way ANOVA to test the effects of the experimental time (1, 3, 7, 14 and 28 days), injected doses of Al_2O_3-NPs (0 and 1.3 g kg^{-1} bw) and their interactions, on the RBC count, Hb content, Hct value, WBC count, blood Plt count and blood indices (MCV, MCH and MCHC) of male albino rats during sublethal experiments.[a]

Source	Sum of squares	*df*	Means of squares	$F_{calculated}$	Significant levels (*p* value)
RBC count					
Experimental time	23.443	4	5.861	15.691	<0.0001
Doses	29.215	1	29.215	78.217	<0.0001
Time/doses interaction	16.641	4	4.160	11.138	<0.0001
Residual (error)	14.941	40	0.374		
Hb content					
Experimental time	88.920	4	22.230	22.506	<0.0001
Doses	51.369	1	51.369	52.008	<0.0001
Time/doses interaction	122.857	4	30.714	31.096	<0.0001
Residual (error)	39.509	40	0.988		
Hct value					
Experimental time	661.090	4	165.273	19.108	<0.0001
Doses	544.170	1	544.170	62.913	<0.0001
Time/doses interaction	744.240	4	186.060	21.511	<0.0001
Residual (error)	345.985	40	8.650		
WBC count					
Experimental time	6.539	4	1.635	3.260	<0.05
Doses	3.120	1	3.120	6.221	<0.05
Time/doses interaction	7.015	4	1.754	3.497	<0.05
Residual (error)	20.061	40	0.502		
Plt count					
Experimental time	58,304.6	4	14576.2	2.214	>0.05
Doses	11852577.5	1	11852577.5	1800.2	<0.0001
Time/doses interaction	58041.9	4	14510.5	2.204	>0.05
Residual (error)	263365.04	40	6584.12		
MCV					
Experimental time	310.235	4	77.559	13.817	<0.0001
Doses	55.440	1	55.440	9.877	<0.01
Time/doses interaction	471.428	4	117.857	20.997	<0.0001
Residual (error)	224.525	40	5.613		
MCH					
Experimental time	11.792	4	2.948	2.912	<0.05
Doses	22.191	1	22.191	21.923	<0.0001
Time/doses interaction	15.045	4	3.761	3.716	<0.05
Residual (error)	40.490	40	1.012		
MCHC					
Experimental time	61.895	4	15.474	7.396	<0.0001
Doses	31.237	1	31.237	14.930	<0.0001
Time/doses interaction	38.266	4	9.566	4.572	<0.01
Residual (error)	83.687	40	2.092		

RBC: red blood cell; Hb: haemoglobin; Hct: haematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular Hb; MCHC: MCH concentration; WBC: white blood cell; Plt: platelet; ANOVA: analysis of variance; Al_2O_3-NP: aluminium oxide nanoparticles.
[a]$p > 0.05$: insignificant effect; $p < 0.05$, $p < 0.01$ and $p < 0.0001$: significant effects at $\alpha = 0.05$, 0.01 and 0.0001, respectively.

Biochemical parameters

The concentrations of TLs, TPs, urea, uric acid, creatinine and the activities of AST and ALT in serum of male albino rats were estimated during the acute and sublethal experiments.

In acute experiments, one-way ANOVA and correlation coefficient revealed that the injected doses of Al_2O_3-NPs (0, 3.9, 6.4 and 8.5 g kg^{-1} bw) significantly affected and decreased the concentrations of the TL and TP but increased the levels of urea, uric acid and

Table 4. The RBC count (10^{12} L^{-1}), Hb (g dL^{-1}), Hct (%), MCV (*fl*), MCH (ρg), MCHC (g dL^{-1}), WBC count (10^9 L^{-1}) and the blood Plt count (10^9 L^{-1}) of control male albino rats (group V) and those injected with 1.3 g Al_2O_3-NP kg^{-1} bw 2 $days^{-1}$ (group VI).[a]

Parameters	Experimental periods (days)					Fitting equations[b]	*r*
	1	3	7	14	28		
RBC count							
Group V	6.9 ± 0.30	6.4 ± 0.27	6.7 ± 0.27	6.3 ± 0.40	6.3 ± 0.09	–	–
Group VI (%Change)[e]	10.3 ± 0.14[c] (+49.9%)	8.6 ± 0.29[c] (+33.9%)	7.8 ± 0.24[d] (+15.1%)	7.2 ± 0.08[d] (+14.5%)	7.0 ± 0.26[d] (+9.3%)	$y = -1.05\ln(x)+10.0$	−0.74
Hb content							
Group V	12.8 ± 0.28	12.4 ± 0.19	11.7 ± 0.49	12.7 ± 0.55	12.6 ± 0.35	–	–
Group VI (%Change) [e]	19.0 ± 0.32[c] (+48.3%)	16.3 ± 0.89[c] (+31.0%)	13.0 ± 0.16[d] (+10.9%)	11.2 ± 0.46[d] (−11.6%)	11.4 ± 0.26[d] (−9.7%)	$y = -2.4\ln(x)+18.7$	−0.76
Hct value							
Group V	31.9 ± 0.79	30.4 ± 0.71	31.9 ± 1.38	31.8 ± 0.23	32.3 ± 0.63	–	–
Group VI (%Change)[e]	50.9 ± 0.81[c] (+59.6%)	41.9 ± 3.45[c] (+37.6%)	34.6 ± 0.56 (+8.5%)	34.4 ± 0.63 (+8.4%)	29.5 ± 0.73 (−8.8%)	$y = -6.3\ln(x)+49.5$	−0.75
WBC count							
Group V	2.9 ± 0.40	3.3 ± 0.37	3.2 ± 0.33	3.3 ± 0.46	3.0 ± 0.37	–	–
Group VI (%Change) [e]	4.5 ± 0.11[f] (+55.2%)	4.4 ± 0.13[d] (+34.8%)	3.4 ± 0.29 (+7.9%)	3.0 ± 0.31 (−7.0%)	2.7 ± 0.19 (−8.7%)	$y = -0.6\ln(x)+4.7$	−0.76
Blood Plt							
Group V	261 ± 5.2	272 ± 20.6	253 ± 6.4	270 ± 13.5	263 ± 2.8	–	–
Group VI (%Change)[e]	1171 ± 43.8[c] (+348%)	1159 ± 45.2[c] (+326%)	1227 ± 43.4[c] (+384%)	1312 ± 64.4[c] (+389%)	1320 ± 50.0[c] (+401%)	$y = -0.19x+39.0$	+0.48
MCV							
Group V	48.2 ± 1.4	48.3 ± 1.1	48.5 ± 1.0	48.9 ± 1.3	49.8 ± 0.9	–	–
Group VI (%Change)[e]	53.3 ± 0.9[f] (+10.69%)	49.8 ± 1.4 (+3.19%)	47.8 ± 0.6 (−1.42%)	45.4 ± 1.3[d] (−7.20%)	36.8 ± 0.6[c] (−26.10%)	$y = 0.21x+261.6$	−0.93
MCH							
Group V	18.1 ± 0.6	19.1 ± 0.4	18.4 ± 0.4	18.6 ± 0.2	18.7 ± 0.3	–	–
Group VI (%Change) [e]	18.4 ± 0.3 (+1.65%)	18.0 ± 0.6 (−5.61%)	17.1 ± 0.6 (−6.87%)	17.1 ± 0.5[d] (−8.16%)	15.6 ± 0.2[c] (−16.69%)	$y = -0.10x+18.3$	−0.69
MCHC							
Group V	39.3 ± 0.8	38.6 ± 0.9	38.4 ± 0.7	38.3 ± 1.0	38.5 ± 0.8	–	–
Group VI (%Change) [e]	40.0 ± 0.2 (+1.73%)	37.5 ± 0.1 (−2.72%)	37.4 ± 0.2 (−2.61%)	36.4 ± 0.6[d] (−4.89%)	33.9 ± 0.5[c] (−12.10%)	$y = -0.19x+39.0$	−0.89

RBC: red blood cell; Hb: haemoglobin; Hct: haematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular Hb; MCHC: MCH concentration; WBC: white blood cell; Plt: platelet; ANOVA: analysis of variance; Al_2O_3-NP: aluminium oxide nanoparticles.

[a]Data represented as a mean of five rats ± SEM.

[b]The fitting equation of the relationship and correlation coefficients (*r*) of the experimental periods (time) with the haematological parameters.

[c]Significant difference in comparison with the corresponding controls at $\alpha = 0.0001$.

[d]Significant difference in comparison with the corresponding controls at $\alpha = 0.05$.

[e]Percentage of changes in comparison with the corresponding controls.

[f]Significant difference in comparison with the corresponding controls at $\alpha = 0.01$.

Table 5. The concentrations of TLs, TP, urea, uric acid, creatinine and the activities of AST and ALT in serum of control male albino rats and those injected with a single acute dose of Al_2O_3-NPs (3.9 g or 6.4 g or 8.5 g kg^{-1} bw) after 2 days of injection. [a]

Experimental groups	TL (mg dL^{-1})	TP (g dL^{-1})	Urea (g dL^{-1})	Uric acid (mg dL^{-1})	Creatinine (mg dL^{-1})	Transaminases (U L^{-1})	
						AST	ALT
Group I	464.94 ± 5.38	6.68 ± 0.09	32.59 ± 1.01	1.18 ± 0.06	1.36 ± 0.10	25.40 ± 0.98	19.60 ± 0.75
Group II [d](%Change)	460.77 ± 2.61 (−0.9%)	5.78 ± 0.23 [b] (−13.5%)	33.97 ± 0.98 (+4.23%)	1.53 ± 0.07[c] (+29.7%)	1.33 ± 0.09 (−2.21%)	49.22 ± 1.18[c] (+94%)	33.15 ± 0.52[c] (+69.1%)
Group III [d](%Change)	318.66 ± 19.59[c] (−31.5%)	4.87 ± 0.07[c] (−27.1%)	34.50 ± 1.29 (+5.86%)	1.88 ± 0.03[c] (+59.3%)	1.42 ± 0.15 (+4.41%)	55.75 ± 1.06[c] (+119%)	34.13 ± 0.33[c] (+74.1%)
Group IV [d](%Change)	301.49 ± 16.64[c] (−35.2%)	4.48 ± 0.15[c] (−32.9%)	37.42 ± 0.65 [b] (+14.82%)	2.25 ± 0.03[c] (+90.7%)	2.17 ± 0.14[c] (+59.56%)	63.20 ± 2.06[c] (+149%)	39.10 ± 0.68[c] (+99.5%)
Effect of doses							
One-way ANOVA (p–value)	$F_{3,16} = 44.99$ (<0.0001)	$F_{3,16} = 41.91$ (<0.0001)	$F_{3,16} = 4.06$ (<0.05)	$F_{3,16} = 71.11$ (<0.0001)	$F_{3,16} = 10.43$ (<0.0001)	$F_{3,16} = 138.5$ (<0.0001)	$F_{3,16} = 198.8$ (<0.0001)
[e]Fitting equation	$y = 494.99e^{-0.057x}$	$y = -0.23x+6.71$	$y = 32.27e^{0.015x}$	$y = 1.6e^{0.076x}$	$y = 1.07e^{0.147x}$	$y = 4.398x+27.72$	$y = 2.196x+21.18$
Correlation coefficient	−0.84	−0.94	+0.60	+0.95	+0.59	+0.96	+0.95

TP: total protein; ANOVA: analysis of variance; Al_2O_3-NP: aluminium oxide nanoparticles; TL: total lipid; AST: aspartate aminotransferase; ALT: alanine aminotransferase.
[a]Each value is a mean of five rats ± SEM.
[b]Significant difference in comparison with the corresponding control at $\alpha = 0.01$.
[c]Significant difference in comparison with the corresponding control at $\alpha = 0.0001$
[d]Percentage changes in comparison with the corresponding controls. $p < 0.05$ and 0.0001: significant effect of doses at $\alpha = 0.05$ and 0.0001 respectively.
[e]Fitting equation: it fits the relationship and correlation coefficient of the injected doses with the studied parameters.

Table 6. Two-ways ANOVA to test the effects of the experimental time (1, 3, 7, 14 and 28 days), injected doses of Al_2O_3-NPs (0 and 1.3 g kg^{-1} bw) and their interaction on the concentrations of the TL, TP, urea, uric acid and creatinine and the activities of AST and ALT in serum of male albino rats.[a]

Parameter	Sum of squares	*df*	Means of squares	$F_{Calculated}$	*p* value
TLs (mg dL^{-1})					
Time	2287.03	4	571.75	4.60	<0.01
Doses	1192273.7	1	1192273.7	9604.4	<0.0001
Time/doses interaction	1026.23	4	256.56	2.067	>0.05
Error	4965.51	40	124.138		
TP (g dL^{-1})					
Time	6.25	4	1.563	17.017	<0.0001
Doses	25.68	1	25.67	279.62	<0.0001
Time/doses interaction	3.773	4	0.943	10.274	<0.0001
Error	3.673	40	0.092		
Urea (mg dL^{-1})					
Time	321.7	4	80.428	17.182	<0.0001
Doses	1193.7	1	1193.7	255.03	<0.0001
Time/doses interaction	526.26	4	131.566	28.107	<0.0001
Error	187.23	40	4.681		
Uric acid (mg dL^{-1})					
Time	0.060	4	0.015	0.614	>0.05
Doses	7.174	1	7.174	295.89	<0.0001
Time/doses interaction	0.031	4	0.008	0.324	>0.05
Error	0.970	40	0.024		
Creatinine (mg dL^{-1})					
Time	0.76	4	0.19	8.83	<0.0001
Doses	0.42	1	0.42	19.46	<0.0001
Time/doses interaction	0.97	4	0.24	11.21	<0.0001
Error	0.86	40	0.02		
AST (U L^{-1})					
Experimental time	58.070	4	14.517	3.437	<0.05
Doses	4780.269	1	4780.269	1131.700	<0.0001
Time/doses interaction	96.063	4	24.016	5.686	<0.01
Residual (error)	168.959	40	4.224		
ALT (U L^{-1})					
Experimental time	23.577	4	5.894	2.284	>0.05
Doses	57.846	1	57.846	22.413	<0.0001
Time/doses interaction	6.788	4	1.697	0.658	>0.05
Residual (error)	103.235	40	2.581		

TP: total protein; ANOVA: analysis of variance; Al_2O_3-NP: aluminium oxide nanoparticles; TL: total lipid; AST: aspartate aminotransferase; ALT: alanine aminotransferase.

[a]$p > 0.05$: insignificant effect; $p < 0.05$, $p < 0.01$ and $p < 0.0001$: significant effect at $\alpha = 0.05$, 0.01 and 0.0001, respectively.

creatinine and the activities of the ALT and AST in serum of male albino rats after 2 days of administration (Table 5). In comparison with the corresponding controls, the concentrations of TP in rats of groups II, III and IV significantly decreased, while the levels of TL markedly depleted in serum of rats of groups III and IV only (Table 5). On the contrary, the concentrations of uric acid and the activities of AST and ALT were increased and became significantly higher than the corresponding controls, at all injected acute doses, whereas the levels of the urea and creatinine were significantly elevated only at the highest-injected dose (group IV) and became higher than the corresponding controls (Table 5). Regression analysis and the correlation coefficient indicated that the injected doses of nanoalumina were inversely proportional to the levels of TL and TP, whereas the concentrations of urea, uric acid, creatinine and the activities of ALT and AST exhibited a direct proportion, with correlation coefficients (r) of −0.84, −0.94, +0.60, +0.95, +0.59, +0.95 and +0.96, respectively (Table 5).

Table 7. The concentrations of TL (g dL^{-1}), TP (g dL^{-1}), urea (g dL^{-1}), uric acid (mg dL^{-1}), creatinine (mg dL^{-1}) and activities of transaminases (AST and ALT, U L^{-1}) in serum of the control male albino rats and those injected with a repeated doses of Al_2O_3-NPs (1.3 g kg^{-1} bw), after 1, 3, 7, 14 and 28 days of injection.[a]

Parameters	Experimental periods (days)					Effect of time (ANOVA)	Fitting equations[a]	r
	1	3	7	14	28			
TLs								
Group V	469.48 ± 6.7	460.37 ± 6.4	458.17 ± 4.5	467.52 ± 5.8	465.09 ± 8.3	$F_{4,20} = 0.55$, $p > 0.05$	–	–
Group VI (% Change) [d]	176.39 ± 2.4[b] (−62.43%)	153.38 ± 2.5[b] (−66.68%)	149.44 ± 3.7[b] (−67.38%)	148.70 4.0[b] (−68.19%)	148.54 ± 0.1[b] (−68.06%)	$F_{4,20} = 17.41$, $p < 0.0001$	$y = 7.84\ln(x)+169.4$	−0.52
Total Protein (TP)								
Group V	6.87 ± 0.13	6.59 ± 0.11	6.84 ± 0.23	6.53 ± 0.13	6.72 ± 0.01	$F_{4,20} = 1.13$, $p > 0.05$	–	–
Group VI (% Change) [d]	6.51 ± 0.02 (−5.24%)	5.00 ±.16[b] (−24.13%)	4.98 ± 0.16[b] (−27.19%)	4.97 ± 0.15[b] (−23.89%)	4.92 ± 0.11[b] (−26.79%)	$F_{4,20} = 29.93$, $p < 0.0001$	$y = -0.425\ln(x)+6.0$	−0.49
Urea								
Group V	34.60 ± 1.06	34.89 ± 1.35	33.92 ± 1.28	33.13 ± 0.81	33.04 ± 1.06	$F_{4,20} = 0.55$, $p > 0.05$	–	–
Group VI (% Change) [d]	35.79 ± 0.33 (+3.44%)	37.96 ± 0.31 (+8.79%)	46.08 ± 0.20[b] (+35.85%)	48.13 ± 1.28[b] (+45.28%)	50.48 ± 1.05[b] (+52.78%)	$F_{4,20} = 69.36$, $p < 0.0001$	$y = 4.8\ln(x)+35.0$	+0.84
Uric acid								
Group V	1.66 ± 0.03	1.65 ± 0.05	1.64 ± 0.04	1.59 ± 0.04	1.68 ± 0.03	$F_{4,20} = = 0.78$, $p > 0.05$	–	–
Group VI (% Change) [d]	2.34 ± 0.06[b] (+40.96%)	2.37 ± 0.08[b] (+43.64%)	2.39 ± 0.06[b] (+45.73%)	2.39 ± 0.15[b] (+50.31%)	2.50 ± 0.08[b] (+48.81%)	$F_{4,20} = 0.42$, $p > 0.05$	$y = 0.01x+2.3$	+0.27
Creatinine								
Group V	1.55 ± 0.04	1.57 ± 0.02	1.50 ± 0.03	1.58 ± 0.02	1.50 ± 0.03	$F_{4,20} = 1.61$, $p > 0.05$	–	–
Group VI (% Change) [d]	1.43 ± 0.05 (−7.74%)	1.45 ± 0.07 (−7.64%)	1.73 ± 0.17[b] (+15.33%)	1.89 ± 0.03[b] (+19.62%)	2.11 ± 0.03[b] (+40.67%)	$F_{4,20} = 10.94$, $p < 0.0001$	$y = 1.36x^{-0.12}$	+0.79
AST								
Group V	25.53 ± 0.74	26.12 ± 0.41	25.15 ± 0.61	24.73 ± 0.69	24.80 ± 0.68	$F_{4,20} = 0.82$, $p > 0.05$	–	–
Group VI (% Change) [d]	41.66 ± 0.23[b] (+63.18%)	43.71 ± 0.14[b] (+67.34%)	44.77 ± 0.60[b] (+78.01%)	44.86 ± 0.46[b] (+81.39%)	49.10 ± 2.40[b] (+97.98%)	$F_{4,20} = 5.75$, $p < 0.01$	$y = 0.24x+42.3$	+0.70
ALT								
Group V	20.74 ± 0.82	20.82 ± 0.81	20.95 ± 0.75	21.98 ± 0.01	21.15 ± 0.94	$F_{4,20} = 0.45$, $p > 0.05$	–	–
Group VI (% Change) [d]	22.08 ± 0.24 (+6.46%)	22.68 ± 0.15 (+8.93%)	22.68 ± 0.57 (+8.26%)	24.33 ± 0.09[e] (+10.69%)	24.62 ± 1.40[b] (+16.41%)	$F_{4,20} = 2.66$, $p > 0.05$	$y = 0.79\ln(x)+21.8$	+0.54

TP: total protein; ANOVA: analysis of variance; Al_2O_3-NP: aluminium oxide nanoparticles; TL: total lipid; AST: aspartate aminotransferase; ALT: alanine aminotransferase.
[a]Each value is a mean of five rats ± SEM.
[b]The fitting equation of the relationship and correlation coefficients (r) of the experimental periods (time) with the studied parameters.
[c]Significant difference in comparison with the corresponding controls at $\alpha = 0.0001$.
[d]Percentage of changes in comparison with the corresponding controls.
[e]Significant difference in comparison with the corresponding controls at $\alpha = 0.05$.

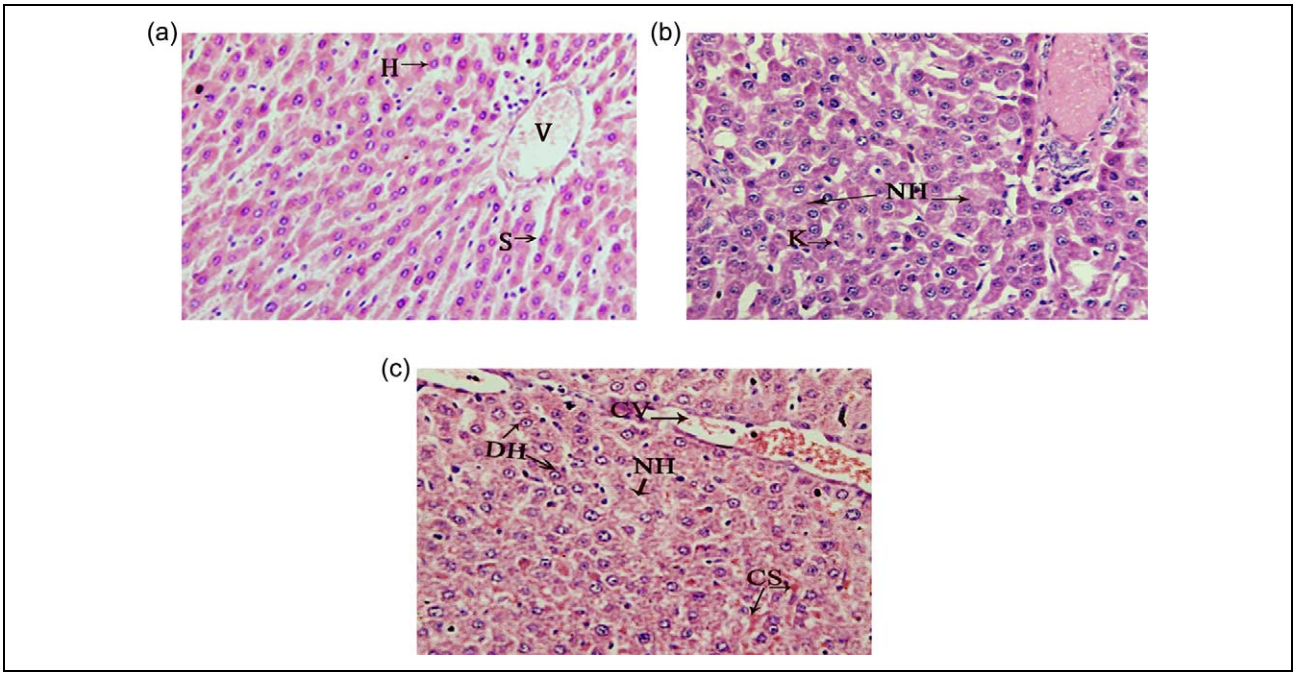

Figure 1. (a) Cross section of the liver of control male albino rat showing the normal hepatocyte (H), central vein (CV) and sinusoids (S) (HE, ×20). (b) Cross section of the liver of male albino rat after 2 days of injection of a single acute dose of nanoalumina (8.5 g kg^{-1}) showing the necrotic hepatocytes (NH) and Kupffer cell (K) (HE, ×20). (c) Cross section of the liver of male albino rat after 28 days of injection of sublethal dose of nanoalumina (1.3 g kg^{-1} 2 $days^{-1}$) showing the congested vein (CV), the disarray of hepatocytes (DH), congested blood sinusoids (CS) and necrotic hepatocytes (NH) (HE, ×20). HE: haematoxylin and eosin.

In sublethal experiments, two-ways ANOVA revealed that the concentrations TL, TP, urea, creatinine and the activities of AST and ALT were significantly affected by the experimental periods, injected doses (0 and 1.3 g) and their interactions, whereas the levels of uric acid were influenced by the injected doses only (Table 6). According to LSD, rats of group VI showed significant decrease in the levels of TL and TP in serum and became lower than their corresponding controls at all the experimental periods, except for the TP after 1 day of injection (Table 7). In addition, the experimental periods showed negative relationship with the levels of TL and TP of group VI with correlation coefficient of −0.55 and −0.49, respectively (Table 7). The concentrations of urea, uric acid and creatinine increased significantly and became greater than the corresponding controls, at all the experimental periods, but the levels of urea and creatinine showed an insignificant increase after 1 and 3 days post-injection (Table 7). In comparison with the corresponding controls, the activity of AST significantly increased, at all the experimental periods, whereas the ALT activity exhibited significant increase, after 14 and 28 days post-injection (Table 7). The regression analysis showed that the experimental periods were directly proportion to the levels of urea, uric acid, creatinine and the activities of AST and ALT, in rats of group VI, with correlation coefficients (r) of +0.84, +0.27, +0.79, +0.70 and +0.57, respectively (Table 7).

Histological examination

In the present study, the control rats and the rats injected with Al_2O_3-NPs did not show any abnormal appearance in their integumentary system, including the skin and fur. Autopsy showed some white spots on the liver lobes, spleen and intestine of rats injected with Al_2O_3-NPs, whereas in the control rats, no abnormalities were observed.

The hepatic tissues of controls showed a normal architecture without histological abnormalities (Figure 1(a)). The hepatocytes (H) arranged in a regular array radiating from the central vein (V) are surrounded by blood sinusoids (S) and characterized by the presence of an obvious of spherical nuclei (Figure 1(a)). In rats of group III, the hepatic tissues exhibited irregular disarray that accompanied by a progressive increase in the necrosis of the hepatocytes (NH) and

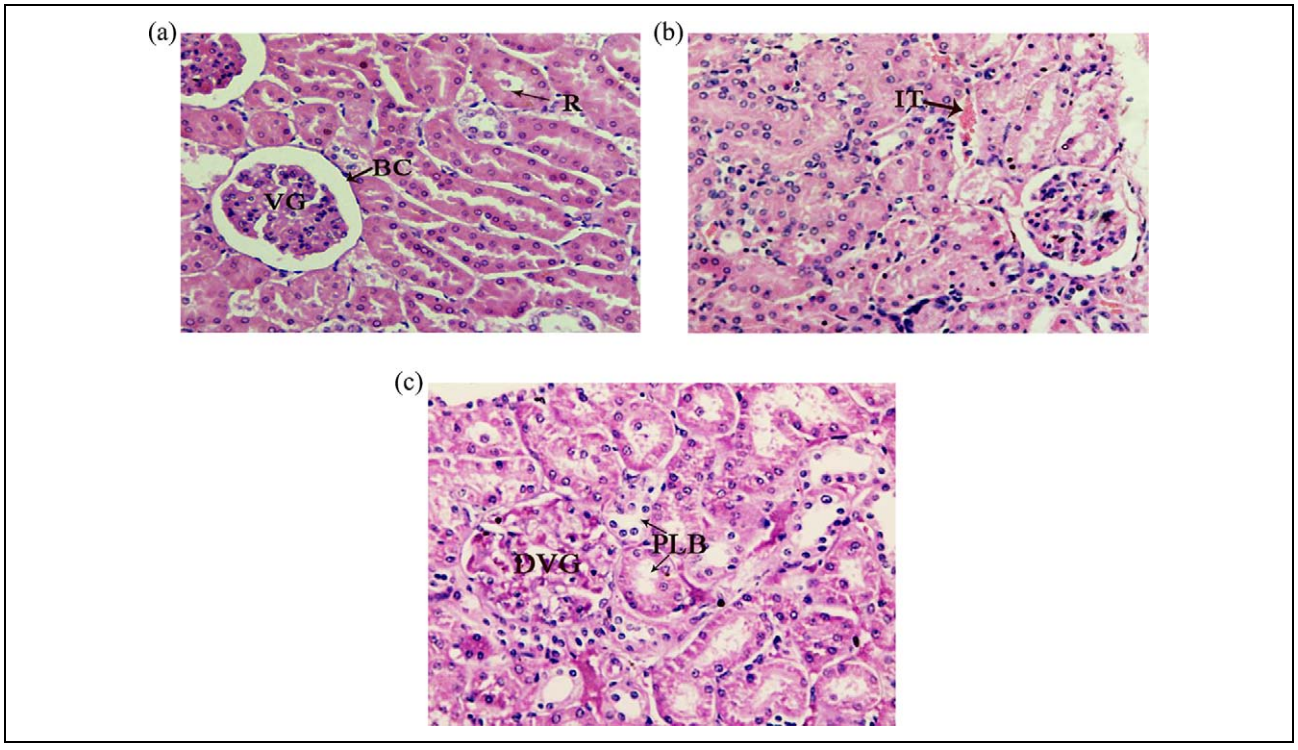

Figure 2. (a) Cross section of the kidney of control male albino rat showing the Bowman's capsule (BC), renal tubule (R) and vascular glomerulus (DVG) (HE, ×20). (b) Cross section of the kidney of male albino rat after 2 days of injection of a single acute dose of nanoalumina (8.5 g kg^{-1} 2 $days^{-1}$) showing the inter-tubular congestion (IT) (HE, ×20). (c) Cross section in the kidney of male albino rat after 28 days of injection of sublethal dose of nanoalumina (1.3 g kg^{-1} 2 $days^{-1}$) showing the dilation of vascular glomeruli (DVG) and the partial loss of brush border (PLB) of the renal tubule. (HE, ×20). HE: haematoxylin and eosin.

Kupffer cells (K) (Figure 1(b)). Throughout the course of sublethal experiments, no histological alterations are observed in the liver of rats injected with Al_2O_3-NPs, after 1, 3, 7 and 14 days post-injection. On the 28th day of injection, the hepatic tissue showed marked severe disarray (DH) that are associated with a congested blood sinusoids (CS) and necrotic hepatocytes (Figure 1(c)).

The normal histological structure of the kidney sample of control is observed in Figure 2(a). After 2 days of acute dosing, there were no histological changes in the renal tissue except for the rats of group IV characterized by the presence of inter-tubular congestion (IT) among the renal tubules (R) (Figure 2(b)). In addition, rats of group VI did not exhibit any valuable remarked histological alterations except the dilation of the vascular glomeruli (DVG) that are completely occupied by the Bowman's capsule (BC) and a partial loss in the brush border (PLB) of renal tubules (Figure 2(c)).

The examination of the cerebral cortex of controls, by the light microscope, showed the normal neurons (N) with obvious nuclei and intact cell membranes (Figure 3(a)). At the end of acute experiments, no histological changes observed in the cerebral cortex of groups II and III, whereas those of group IV were characterized by a marked progressive degeneration in the brain's neurons (DN) (Figure 3(b)). In addition, throughout the sublethal experiments, no histological alterations were detected in the cerebral cortex of rats of group VI, at all the experimental periods except at the 28th day post-injection, at which a large number of degenerated neurons were observed (Figure 3(c)). Degenerated neurons were characterized by eosinophilic staining of both cell body and proximal dendrites when compared with the healthy neurons that exhibit haematoxylin-stained nuclei with a lightly eosin-stained perinuclear cytoplasm.

Discussion

Haematological parameters

The RBC count was not affected by the acute injected doses in rats of groups II, III and IV and did not differed significantly with the corresponding controls, whereas the MCV was significantly higher than the controls

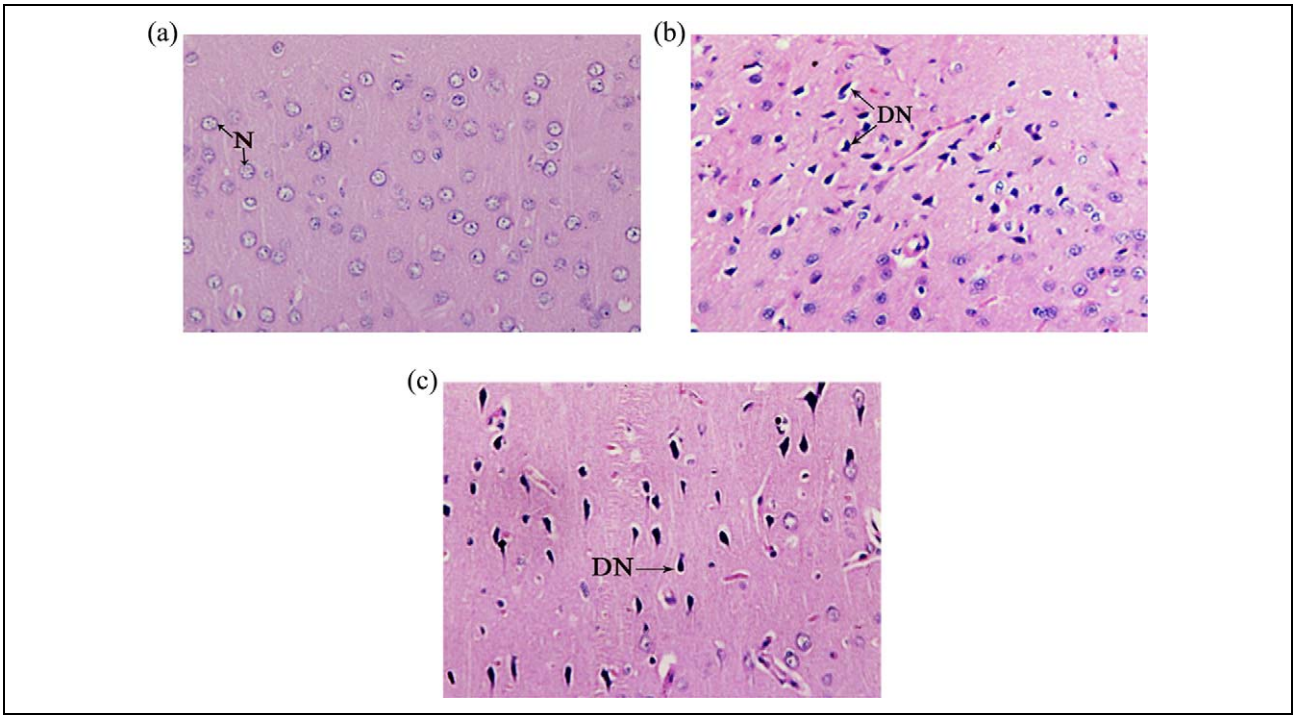

Figure 3. (a) Cross section of the cerebral cortex of control male albino rat showing the normal structure of neuron (N) (HE, ×20). (b) Cross section of the cerebral cortex of male albino rat after 2 days of injection of a single acute dose of nanoalumina (8.5 g kg^{-1}) showing the marked increase in the number of degenerated neurons (DN) (HE, ×20). (c) Cross section of the cerebral cortex of male albino rat after 28 days of injection of a sublethal dose of nanoalumina (1.3 g kg^{-1} 2 $days^{-1}$) showing the degeneration of neurons (DN) (HE, ×20). HE: haematoxylin and eosin

(macrocytosis) and exhibited a positive correlation with the injected doses. The insignificant changes in RBC count may be attributed to the amounts of Al accumulated by the erythrocytes (during 2 days), which were not sufficient to initiate significant effects on the RBC count (Ali, 2013). The demonstrated macrocytosis can be suggested by the enlargement of the RBC count as a result of Al accumulated in the erythrocytes and not due to the Hb that depleted during our acute experiments, causing a marked increase in their size. Ali (2013) reported that the nanoalumina, due to their nanosize (<13 nm), are highly able to penetrate the cell membranes and accumulate inside the cells, such as RBCs, leading to increase their size and volume (macrocytosis). In addition, the macrocytosis may be considered as a haemodilution mechanism to reduce and overcome the irritation of erythrocyte through indirect dilution of Al invading these cells (Bhagwant and Bhikajee, 2000). The nuclear DNA damage, induced by Al injection (Ali, 2013), caused the splitting of DNA strands that caused alteration in amino acids sequence, leading to the production of abnormal large erythroblasts with nuclear/cytoplasmic asynchrony in the bone marrow (Aslinia et al., 2006).

In rats of group VI, at all periods of the experiments, in spite of the RBC count being significantly higher than the corresponding controls, the experimental periods showed a negative correlation with the RBC count and MCV with correlation coefficient of −0.74 and −0.93, that is, RBC count and MCV are time dependent. During the sublethal experiments, the demonstrated significant increase in RBC count, in comparison with the related controls, was temporary because the experimental times were not enough to induce RBC count depletion, in rats of group VI. This can be proved using the fitting equation, $[y = -1.05\ln(x) + 10]$ for RBC count, as shown in Table 4, and by substituting x (time in days) with 60 and 90 days, which will give output y (RBC count) as 5.7 and 5.2 g Al kg^{-1} dry tissue, respectively. This indicated that the RBC count significantly decreased with increasing experimental periods, that is, the injection of nanoalumina decreases the RBC count. The endogenous antioxidants inside the RBCs (Milovanović et al., 2012) are highly effective against Al-oxidative stress, resulting in increasing the lifespan of RBCs in the circulation. Al accumulated in the renal tissues caused a regional renal hypoxia that enhanced and accelerated the synthesis of the ectopic erythropoietin (Epo) (Chmielnicka et al.,

1994). The Epo promotes the viability, proliferation and differentiation of the mammalian erythroid progenitor cells through their control on the ectopic Epo receptor (EpoR) (Verdier et al., 2000) and functions as an anti-apoptotic factor in order to maintain the cells alive and reach their maturity (Koury et al., 2002).

The microcytic anaemia (microcytosis), significant decreases in MCV after 14 and 28 days of injection, may be suggested by the depletion of Hb content inside the RBCs, disturbance in the erythropoiesis in the medullary and extra-medullary sites (e.g. the spleen) as a result of hypoxia induced by Al toxicity and the deficiency of the folic acid and cobalamin (vitamin B12). The medullary and extra-medullary stress activates the haematopoiesis (Lenox et al., 2009) by the induction of bone morphogenetic proteins-4, the stem cell factor and the hypoxia to initiate and promote the proliferation and differentiation of the earliest erythroid progenitor, the burst forming unit-erythroid to new mature and immature small sizes erythrocytes, microcytic anaemia, as a physiological adaptation to overcome the hypoxia (Perry et al., 2007). In addition, Al accumulated in the gastrointestinal tract (Ali, 2013) may compete with the cobalamin (vitamin B12) to use the H2-histamine blockers leading to the atrophy or loss of the gastric mucosa that will not be able to synthesize and secret the intrinsic factor that inhibits the absorption of the folic acid and cobalamin necessary for the process of erythropoiesis causing the megablastic anaemia (Ahmed et al., 2011). The Hct values of groups III, IV and VI (after 1 and 3 days) were significantly higher than the corresponding controls. It is well known that the values of Hct depend mainly on the values of MCV and RBC count (Shah, 2006). Therefore, the recorded increase in Hct values could be related to the elevated MCV, during acute experiments and increased RBC count, during sublethal experiments.

The significant decrease in the concentrations of Hb in rats of groups III, IV and VI may be attributed to the oxidation of the Hb at the molecular level and the fail in the process of haem-biosynthesis due to the shortage of the components, such as proteins, iron and enzymes, necessary for Hb synthesis. In the present data, Al accumulated in the liver caused a marked damage to the hepatic tissues by the reactive oxygen species (ROS) (Ali, 2013), leading to a marked depletion in the TP contents and shortage of the globin proteins required for Hb synthesis. Due to high surface tension and hyperactivity of nanoalumina, the outer layer of the metal liberates Al^{3+} (Burklew et al., 2012). The liberated Al^{3+} expels the Fe^{3+} from the tissues and molecules, especially the transferrin (Mahieu et al., 2000) and inhibits the ceruloplasmin, which promotes the incorporation of the ferric ion into the transferrin (Chmielnicka et al., 1994), leading to a decrease in the uptake of Fe^{3+} necessary for Hb synthesis and consequently causing mild anaemia (Flora et al., 2003). In addition, the accumulation of Al in haematopoietic tissues could inhibit the activities of enzymes necessary for the process of haem-biosynthesis such as δ-aminolevulinic acid dehydratase (δ-ALAD) (Pimentel-Vieira et al., 2000) and haem synthetase (Chmielnicka et al., 1994), leading to the reduction in Hb synthesis and to the hypoxia as a result of reduced O_2 carrying capacity by Hb. At the beginning of the sublethal experiments, the significant increase in the Hb, after 1, 3 and 7 days post-injection, may ascribed to the positive feedback mechanism against the hypoxia, caused by Al accumulated in the RBCs, and enhanced the haem-biosynthesis to increase the bioavailability of Hb to capture more oxygen and to compensate the shortage of the blood oxygen (Peuranen et al., 2003).

The marked reduction in MCH and MCHC in rats of groups II, III, IV and VI could be attributed to the reduction in the Hb content (Kori-Siakpere et al., 2009), which is a result of nanoalumina administration. Moreover, the significant reduction in the MCHC may be suggested by the abnormal swelling of the erythrocytes (Alwan et al., 2009) as confirmed by increased MCV.

The present data revealed that blood Plt count of groups IV and VI were significantly higher than the corresponding controls. The accumulation of Al in the blood Plts and the liver decreases the cholesterol synthesis by the inhibition of 3-hydroxy-3-methyl-glutaryl-CoA reductase (Jope and Johnson, 1992) and minimizes the cholesterol–phospholipids ratio (Silva et al., 2002), leading to an increase in the fluidity of plasma membranes, which improves and increases the lifespan of the blood Plt s in the circulation (Van Rensburg et al., 1992). The excessive overproduction of the ROS, lipid peroxidation (LPO) (Ali, 2013) and hypo-proteinemia, caused by the Al accumulated in blood Plts, activated the blood Plts lipoxygenase causing an intensive aggregation of the thrombocytes (thrombosis) in the blood circulation (Neiva et al., 1997).

The WBC count of groups II, III, IV and VI (after 1 and 3 days) was significantly higher than the corresponding controls and begun to decrease significantly (leucopenia) after 14 and 28 days. In acute experiments, the positive correlation between the injected

acute doses and the WBC count indicated that the WBC count is dose dependent. Generally, the recorded elevation of WBC count (leucocytosis) may be attributed to the overproduction of leucocytes by haematopoietic tissues as an immune response to the inflammation caused by the oxidative stress of the ROS due to Al accumulation in various tissues (Kori-Siakpere et al., 2006; Warheit et al., 2007). The Al accumulated in the extra-medullary haematopoietic tissues, such as the spleen (Ali, 2013), following nanoalumina injection, boosts the overproduction of the total leucocytes, as intrinsic immune response against nanoalumina toxicity (Kwon et al., 2009). On the other hand, the negative relationship between the experimental time and WBC count and the recorded leucopenia (after 14 and 28 days) can be suggested by the shortage of the components necessary for the synthesis of WBCs such as proteins and lipids that significantly decreased, in our present data, due to nanoalumina toxicity. The present data are consistent with that reported by Srinivas et al. (2011). They reported two- to threefold increase in the total leucocyte count, after 1 and 2 days, following exposure to cerium oxide nanoparticles for 4 hours, as compared to controls, and they attributed that to the inflammatory response stimulated by nanoparticles. Kwon et al. (2009) showed a significant increase in the WBC count of mice, after 4 weeks of inhalation of the fluorescent magnetic nanoparticles.

Biochemical parameters

In the acute and sublethal experiments, the levels of TL and TP decreased significantly, in comparison with the corresponding controls, and were associated with a marked increase in the concentrations of creatinine, urea, uric acid and the activities of AST and ALT in serum of rats-injected with Al_2O_3-NPs, in dose- and time-dependent manner.

The decrease in serum TL content may be attributed to the accumulated Al that enhanced the overproduction of free radicals and alter the endogenous antioxidants, resulting in increased LPO, as a direct damage to the lipids produced by the liver, as confirmed by a positive correlation between the concentrations of accumulated Al and malondialdehyde, in the hepatic tissues (Ali, 2013). Al may disturb the lipid metabolism in the hepatocytes (El-Demerdash, 2004), leading to decrease the levels of serum TLs, as confirmed by the negative relationships between the administered doses of Al_2O_3-NPs and the levels of TL, in our data. Moreover, the Al accumulation in the hepatocytes could lead to excessive secretion of catecholamine and corticosteroids causing an excessive utilization of lipids as an extra source of energy to confront the stress caused by Al toxicity (Zaghloul et al., 2002). In addition, Al may cause the impairment of renal functions (Mahieu et al., 2003), as confirmed by increased levels of urea, uric acid and creatinine, leading to the enhancement of lipid excretion through the kidneys and depleting its level in the serum.

The significant depletion of serum TP content may be attributed to the excessive utilization and consumption of albumin as an antioxidant, for the scavenging of free radicals to protect blood cells against oxidative damage (Tolia et al., 2013). This may be related to the accumulation of Al, in the liver, that may interrupt the protein synthesis by damaging the rough endoplasmic reticulum by the ROS (Kutlubay et al., 2007) and consequently reduce the levels of serum TPs. Al may also induce DNA damage in the hepatocytes (Ochmanski and Barabasz, 2000) and cause a disturbance in the amino acids sequence in the strands of the messenger RNA (Hamad, 2012) and alter the process of the protein synthesis, as confirmed, by the direct relationship between the Al accumulated and percentage of DNA damage (Ali, 2013). Moreover, Al may causes the degradation of the plasma proteins by induction of ROS generation (Mostafa, 2009) or/and the enhancement of lysosomal endopeptidases and cathepsins activities (Hamed, 2006) resulting in a marked reduction in the levels of serum TPs.

The increase in the levels of serum uric acid, may be attribute to the renal insufficiency and the reduced renal excretion (Kang et al., 2002), and consequently fail to excrete the uric acid that will be elevated in the blood stream. This may be ascribe to the enhancement of the catabolism of purine bases of DNA, as confirmed by direct relationship between the Al accumulation and comet parameters of the brain (Ali, 2013). In addition, the serum uric acid may be elevated to function as an antioxidant against the excessive production of free radicals (Ashtari et al., 2013). The elevation in urea may also be suggested by the enhancement of protein catabolism (Mahieu et al., 2009), and this is confirmed by the significant reduction in serum TPs, in the present study. The metabolic disturbances such as cations–anions imbalance caused by the Al may lead to alterations in the levels of serum urea (Silva and Goncalves, 2003).

The significant increase in serum creatinine content may be interpreted by the decrease in muscle

mass (Atanasova-Goranova et al., 1997), as a result of enhanced creatine phosphate catabolism to release energy required to withstand Al toxicity (Perrone et al., 1992). The levels of creatinine could reflect the abnormal glomerular filtration rate due to the Al accumulation (Abdel Aziz and Zabut, 2011), as confirmed by the abnormal dilation of vascular glomeruli observed by histological examination of the kidneys, in the present study.

Serum transaminases, ALT and AST. Inside the hepatocytes, Al accumulation may alter the phosphate and ATP metabolism (Silva et al., 2005), leading to the depletion of cellular energy, leading to a disturbance for the membrane potential leading to the necrosis, as confirmed by our histological examination, which may cause the leakage of transaminases into the circulation. As reported by Ali (2013), Al increased the lipid oxidation by the ROS, causing the loss of cell membrane integrity and increased permeability (Nehru and Anand, 2005), resulting in the liberation of AST and ALT into the circulation, as supported by direct relationship of the Al accumulation and levels of MDA in the liver (Ali, 2013). However, rats of group VI showed significant increase in AST activity associated with insignificant change in ALT, after 1, 3 and 7 days of injection. It is known that ALT is found mainly in the cytosol of the hepatocyte and in low levels elsewhere, whereas AST has cytosolic and mitochondrial forms and is distributed in most tissues such as the liver, heart, skeletal muscle, kidneys, brain, pancreas and lungs and in WBCs and RBCs (Giboney, 2005).

Our data are in agreement with several previous studies. El-Demerdash (2004) reported a significant decrease in the concentrations of TL and TP associated with a marked elevation in the levels of urea and activities of AST and ALT, in the serum of male Sprague-Dawely rats, after oral administration of 34 mg of micro-size aluminium chloride ($AlCl_3$) for 30 days, in comparison with the controls. Rajasekaran (2000) reported that the daily oral administration of 260 mg micro-size Al kg^{-1} bw to pubertal male rats for 30 days found to significantly decrease the levels of serum TPs. Hamed (2006) reported a significant decrease in serum TP content of mice, after oral administration of 387.5, 775 and 1550 mg kg^{-1} bw of aluminium sulphate in a dose-dependent relationship after 3 weeks. Newairy et al. (2009) reported that the administration of $AlCl_3$ in male albino rats resulted in increased AST, ALT, urea and creatinine. Viezeliene et al. (2011) reported a significant increase in activity of ALT in serum of mice, after 16 h of injection of 25 mg of $AlCl_3$ kg^{-1} bw. In addition, Bhadauria (2012) demonstrated a significant increase in the activities of AST, ALT and the levels of uric acid and urea in serum of rats, after 24 h of injection with 32.5 mg Al kg^{-1} bw for 3 days. On the other hand, Pasupuleti et al. (2012) reported an inverse dose-dependent increase in the serum activities of AST and ALT of Sprague-Dawley rats, after oral administration of 0.05, 0.3, 1.0 and 2.0 g kg^{-1} bw zinc oxide (ZnO) nanoparticles, 20 nm, at the 14th post-administration. They linked the elevation of AST and ALT activity in serum with the highest magnitude of lesions in the liver, at the low doses of nanoparticles. They ascribed the toxicity of ZnO nanoparticles to the low levels of these NPs accumulated in the tissues at low doses resulting in less agglomeration, making them more able to penetrate into the cells.

Histological changes

The brain of rats administered with Al_2O_3-NPs showed excessive degeneration of neurons in the cerebral cortex in rats of groups IV and VI after 2 and 28 days, respectively. The damage of neurons in the cerebral cortex may be related to the accumulation of Al to a critical level enough to cause the neural degeneration. The blood–brain barrier (BBB) is a protector against the metals penetration (Zheng et al., 2003), but the Al can penetrate it. Al is highly able to penetrate the BBB via the axons and dendrites of neurons (Nel et al., 2006), by direct competition with iron on the transferrin (Yokel et al., 2002) and lactotransferrin (Leveugle et al., 1994) receptors, leading to transport Al instead of iron necessary for the process of myelin formation (Roos et al., 2006). The excessive accumulation of Al and the depletion of iron caused impairment and the vulnerability of neurons to damage. In addition, the nanosized Al is highly able to oxidize the neural membrane that lost its lipoprotein integrity (Banks et al., 2006) and partially cause a damage to the BBB (Yang and Watts, 2005) and in turn facilitates the entrance and accumulation of Al to the brain tissues. The accumulated Al attach to the mitochondria or/and the nuclei causing the damage of cell components at the cellular, subcellular and molecular levels (Chen et al., 2008) and caused degradation of neurons, increased LPO, extent of DNA damage and reduced level of TPs. Al could impair the process of cellular transport mechanisms

(Bizzi et al., 1984), reduce glucose utilization (Joshi, 1990), inhibit phosphorylation–dephosphorylation reactions (Cordeiro et al., 2003) and alter the rate of transmembrane diffusion and selective changes in saturable transport systems in the BBB that may directly lead to the death of neurons (Kaya et al., 2003). However, Al may indirectly affect the neurons by induction of apoptosis in astrocytes (Aremu and Meshitsuka, 2005), which are essential for maintaining neuronal health (Abbott et al., 2006), so any loss or/and damage in astrocyte function could be toxic to neurons. In addition, Al can induce Fe^{2+}-dependent LPO (Zatta et al., 2003), leading to the disruption of lysosomes and liberation of lysosomal enzymes (Britton et al., 2002), causing degeneration of the neurons. The Al could induce LPO in the brain leading to neurodegenration (Kumar et al., 2008; Tripathi et al., 2009), as confirmed by the increased levels of MDA and inhibition SOD, CAT and GPx activities as well as concentrations of GSH in the brain (Ali, 2013). From the above mentioned, it is not surprising that Al has been widely proposed as a main hazard factor in neurodegenerative diseases such as Alzheimer's disease (Walton, 2009), being associated with degenerating neurons in specific central nervous system regions. Our observations are in agreement with Bhadauria (2012) who observed degenerated (pyknotic) neurons in the cerebral cortex, after 3 days of single administration of Al nitrate at a dose of 32.5 mg kg^{-1} (half of LD_{50}). They attributed that Al accumulation in the brain leads to increased rate of phospholipids peroxidation (Newairy et al., 2009) and cell membrane damage and neuron death.

The liver of groups IV and VI showed hepatic disarray that was associated with excessive appearance of Kupffer cells and excessive necrosis of the hepatocytes, but the hepatic disarray was associated with irregular appearance, congested blood sinusoids and necrotic hepatocytes, after the 28 days post-injection. Because the Kupffer cells have a high tendency for the recognition and clearance of xenobiotic, they will in turn recognize the nanocarrier targets (Garnett and Kallinteri, 2006). Therefore, the increased number of Kupffer cells may be attributed to the ability of nanoparticles to evoke the immune responses and enable their clearance (Yen et al., 2009). The observed hepatic necrosis may be attributed to the overproduction of ROS, as a result of Al toxicity, that cause an impairment of the endogenous antioxidant system, with subsequent increase in the LPO (Siddique et al., 2011), associated with cellular damage. The observed hepatic disarray and the irregular colour of the hepatic cells may be attributed to cell–cell dissociation (Inumaru et al., 2009), due to nanoalumina that could reduce the expression of tight junction proteins between the cells (Chen et al., 2008). The observed congestion of blood sinusoids may be related to the ability of Al_2O_3-NPs to induce expression of inflammatory molecules such as intercellular adhesion molecule-1, interleukin-8 and monocyte chemotactic protein-1 (Gojova et al., 2007) and several adhesion molecules (Oesterling et al., 2008), leading to the dysfunction of the luminal endothelium of the sinusoid that is followed by congestion. Our observations are in agreement with several previous studies. Abdelhalim and Jarrar (2011) reported that the gold nanoparticles (Au-NPs) caused infiltration of inflammatory cells after 7 days of administration in rats. Liu et al. (2011) observed lymphocytic infiltration, microgranulation and degenerative necrosis of hepatocytes in the liver of mice after intravenous injection with silica nanoparticles. Stacchiotti et al. (2006) reported abnormal iron deposition, periportal fibrosis and Kupffer cells involvement in the liver of rats after oral administration of Al sulphate.

The kidneys play a major role in preventing the harmful accumulation of Al (Stoehr et al., 2006), by enhancing its excretion from the body through urine. Different mechanisms of renal excretion of Al such as glomerular filtration (Yokel and McNamara, 1985), tubular reabsorption of filtered Al and secretion in distal nephron (Shirley and Lote, 2005) and excretion in the distal tubules (Monteagudo et al., 1988) have been suggested. During the acute experiments, rats of group IV characterized by the presence of inter-tubular congestion among the renal tubules but those of group VI showed the enlargement of vascular glomeruli that completely occupied the Bowman's capsule, as well as a partial loss in the brush border of renal tubules, after the 28 days post-injection. The altered brush border of renal tubules may be attributed to the ability of Al to disrupt the lysosomal membranes leading to the liberation of the oxidative lysosomal enzymes that damage the brush border (Stacchiotti et al., 2002). The observed inter-tubular blood congestion may be attribute to the decrease in the vascular resistance of the renal tissue (Abdelhalim and Jarrar, 2011) induced by nanoparticles. In addition, congestion may be attribute to the accumulation of uric acid crystals in the kidneys leading to the activation of renin angiotensin system, leading to increase the systemic pressure (Kang et al., 2002), which may

cause blood storage in the renal tissue and causing severe congestion. The enlargement of vascular glomeruli could be attributed to the thickening of the glomerular basement membrane to form a barrier against nanoparticles invasion and accumulation (Abdelhalim and Jarrar, 2011). Another possible explanation may be through the ability of nanoparticles to enhance proliferation of mesangial and epithelial cells of Bowman's capsule (Terentyuk et al., 2009). The present results are in agreement with Abdelhalim and Jarrar (2011), who reported occasional inter-tubular blood capillaries dilation and haemorrhage with absence of the proximal tubular brush border in the kidneys of rats that received Au-NPs (10 and 20 nm) for 7 days. On the other hand, the data are in disagreement with Prabhakar et al. (2012) who reported no histological changes in the brain, liver and kidneys of rats after 14 days of oral intubation of single dose of 0.5, 1.0 and 2.0 g Al_2O_3-NPs kg^{-1} bw except for dilation in hepatic central vein. This may be attributed to the doses used in the previous study was lower than our doses as well as the different route of administration.

Conclusion

From the above mentioned points, we can conclude the following:

1. The toxicity of nanoalumina is dose and time dependent.
2. The administration of nanoalumina caused a severe damage to the brain, hepatic and the renal tissues and consequently induced a significant dysfunction for theses organs.
3. As a result of tissue damage, nanoalumina is able to induce some haematological and biochemical disorders.

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