Journal of Pharmacology and Toxicology

ISSN 1816-496X

Academic Journals Inc.

www.academicjournals.com

OPEN ACCESS Journal of Pharmacology and Toxicology

ISSN 1816-496X
DOI: 10.3923/jpt.2020.22.35

Research Article
Intraperitoneal Acute Toxicity of Aluminum Hydroxide Nanoparticle as an Adjuvant Vaccine Candidate in Mice

[1]Sjaikhurrizal El Muttaqien, [1]Etik Mardliyati, [1]Silmi Rahmani, [1]Sabar Pambudi, [1]Siti Maimunah, [2]Ziah Izati Azkia, [2]Nurmeilis, [1]Asri Sulfianti, [1]Julham Efendi, [1]Damai Ria Setyawati, [1]Asutiati Nurhasanah and [1]Irvan Faizal

[1]Centre for Pharmaceutical and Medical Technology, Agency for the Assessment and Application of Technology, Pusat Penelitian Ilmu Pengetahuan dan Teknologi, 15314 Serpong, Indonesia
[2]Department of Pharmacy, State Islamic University Syarif Hidayatullah, 15412 Jakarta, Ciputat, Indonesia

Abstract

Background and Objective: Aluminum-containing adjuvant, such as aluminum hydroxide $Al(OH)_3$), has been widely used and is generally regarded as safe to use in human vaccines. It is known that particle size of adjuvant plays critical role in dictating the adsorption of antigen. Numerous researches reported that down-sizing of $Al(OH)_3$ into nano-sized particles could improve its immunopotentiation character. However, the safety of $Al(OH)_3$ nanoparticle needs to be evaluated through acute toxicity study. This study aimed to assess biological character of nano-sized $Al(OH)_3$, particularly its *in vivo* acute toxicity. **Materials and Methods:** Three types of samples including commercial $Al(OH)_3$ of Alhydrogel®, $Al(OH)_3$ microparticle (Mp) and $Al(OH)_3$ nanoparticle (Np) were prepared and then peritoneally injected to BALB/c mice with 4 different doses (0.16, 1.28, 10.84 and 81.92 mg kg^{-1} b.wt.). Post-administration, mice's behavior and their body weight were recorded. After 14 days, mice were sacrificed and major organs were taken out for preparing hematoxylin-eosin (H and E) stains. **Results:** The average particle size of Alhydrogel®, $Al(OH)_3$ Mp and $Al(OH)_3$ Np were 4.02, 4.21 and 0.162 µm, respectively. The acute toxicity study showed that there is no lethal effect, clinical sign related complications and behavioral alteration even in highest administered dose for 14 days of observation period. In general, histological parameter of H and E stained tissue section among the treated groups was also comparable to those of control group, revealing relatively safe character of $Al(OH)_3$ Np after peritoneal administration. **Conclusion:** Therefore, these results confirmed the potency and safety of $Al(OH)_3$ Np as an adjuvant vaccine candidate.

Key words: Aluminum hydroxide ($Al(OH)_3$), adjuvant, nanoparticle, acute toxicity, human vaccine, immunopotentiation, immunogenesis

Citation: Sjaikhurrizal El Muttaqien, Etik Mardliyati, Silmi Rahmani, Sabar Pambudi, Siti Maimunah, Ziah Izati Azkia, Nurmeilis, Asri Sulfianti, Julham Efendi, Damai Ria Setyawati, Asutiati Nurhasanah and Irvan Faizal, 2020. Intraperitoneal acute toxicity of aluminum hydroxide nanoparticle as an adjuvant vaccine candidate in mice. J. Pharmacol. Toxicol., 15: 22-35.

Corresponding Author: Sjaikhurrizal El Muttaqien, Centre for Pharmaceutical and Medical Technology, Agency for the Assessment and Application of Technology, Pusat Penelitian Ilmu Pengetahuan dan Teknologi, 15314 Serpong, Indonesia

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Aluminum salts, including $Al(OH)_3$, have been extensively used as an adjuvant since past decades to amplify the humoral immunogenicity of the administered vaccine as was first described by Glenny *et al.*[1], Gupta *et al.*[2] and Gupta[3]. This type of adjuvant hypothetically works by providing an antigen local reservoir that could prolong the exposure of antigen to the antigen-presenting cells (APCs) and thus stimulating the activation of immune competent component[4-6]. This depot theory, however, has been questioned as the direct correlation of antigen-$Al(OH)_3$ complex retention at the administration site and the resulting antibody response has never been demonstrated[5,7-9]. Despite the persisted controversy of molecular mechanism of alum adjuvanticity, various attempts to improve the immune response of $Al(OH)_3$ gel have been done by altering the physicochemical properties of adjuvant that can increase the adsorption of the antigen onto the surface of $Al(OH)_3$ gel. For example, recent studies have shown that the modification of shape, dimensions and surface charge of the adjuvant particles critically affect its immune responses[10-14]. In our previous work, $Al(OH)_3$ Nps were successfully prepared with the particle size around 200 nm[15] and its potency was evaluated as adjuvant for potentiating the immune response of purified DENV3 pre Membrane Envelope (prM-E) recombinant protein as an antigen[16]. These studies revealed that the complexes of $Al(OH)_3$ Np-prM-E significantly enhanced immune response compared to those of commercial and micro-sized $Al(OH)_3$ through the induction of nitric oxide release from mouse macrophage RAW264.7cells. Nitric oxide was regarded as an effecter molecule that is involved in macrophages activation and become an important parameter of immune stimulatory activity of the adjuvant[17-19].

In terms of its toxicity, $Al(OH)_3$ adjuvants are generally well tolerated and display favorable safety profile which was indicated by its relative rarity of side effect[20]. Also, previous studies concluded that no clear associations between vaccinations using aluminum-containing vaccines and serious adverse events as 66-70% of injected aluminum was excreted from the body[21] within 24 h. However, it is common that the down-sizing of particle size may alter not only biological activity but also intrinsic toxicity profile of its bulk material. So, the current study was aimed to evaluate the acute toxicity of $Al(OH)_3$ Np and determine its potential risk.

MATERIALS AND METHODS

The animal study was conducted in May-April 2019 in Animal and Pharmacology Laboratories, Center for Pharmaceutical and Medical Technology, BPPT, PUSPIPTEK, Serpong. All animal experiments were approved by the Animal Care and Use Committee of University of Indonesia and were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals as stated at University of Indonesia.

Materials: The $Al(OH)_3$ Mps and NPs were prepared by reacting aluminum chloride with sodium hydroxide, as previously reported by Mardliyati *et al.*[15]. As a stabilizer, sodium polyphosphate (0.2%) was added to $Al(OH)_3$ Np suspension to avoid particle aggregation. Alhydrogel® was purchased from *in vivo* gen (Frederikssund, Denmark). Phosphate-buffered saline (PBS) pH 7.4 was prepared by dissolving one PBS Tablet from Merck in 200 mL of deionized water, yielding 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 SM sodium chloride, pH 7.4.The size and morphology of $Al(OH)_3$ was measured by static light scattering (Horiba LA95A)and transmission electron microscopy/TEM (JEOL F200). The isoelectric point (PI) of each sample was evaluated by Malvern Zetasizer Nano ZS90 equipped with autotitrator mpt-2.

Animals: A total of 65 BALB/c mice (female, 4 weeks old) were obtained from Indo Anilab Laboratories Indonesia, Inc. (Bogor, Indonesia). All animal experiments were approved by the Animal Care and Use Committee of University of Indonesia and were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals as stated at University of Indonesia. The animals were housed in aluminium cages in a well-ventilated room with maintained temperature (20±1°C) and relative humidity (40-70%). Conventional laboratory diet was provided twice daily with unlimited supply of hygienic drinking water. All animals were acclimatized under observation for 1 week before starting the test.

Acute toxicity protocol: The protocol was adopted from the previous intraperitoneal study of acute toxicity conducted by Tubaro and coworkers with slight modification[22]. The animals were randomly divided into 4 groups (5 mice each) as follow: PBS control group, Alhydrogel®, $Al(OH)_3$ Mp and $Al(OH)_3$ Np. For the $Al(OH)_3$ treated group, four different doses were used: 0.16, 1.28, 10.24 and 81.92 mg kg^{-1} b.wt. The lowest dose used in this experiment was obtained from the conversion of recommended $Al(OH)_3$ dose on human (0.2-0.8 mg)[23]. After intraperitoneal administration of the corresponding sample, animal behavior, toxicity sign, body weight and lethal response were observed daily for14 days. At the end of observation period, final body weight of mice was recorded. Mice then were sacrificed after ether anesthesia and their major organs were excised and accurately weighed to measure organs mass index.

Histopathological study: For histological examination, the procedure explained by Slaoui and Fiette[24] was used. The collected lung, liver, kidney and spleen were first fixed in 10% neutral buffered formalin for 24 h at room temperature. Following fixation, the collected tissues were dehydrated, embedded in paraffin, sliced using microtome and placed onto glass slides and stained by hematoxylin and eosin (HE) stain. The slides were then observed using an optical microscope.

Statistical analysis: Result was expressed as mean±standard deviation (SD). The statistical significance was determined using one-way analysis of variance (ANOVA) with the alpha level for all tests were set at $p<0.05$.

RESULTS

Material characterization: The particle size distribution and morphology of $Al(OH)_3$ were evaluated by particle size analyzer and TEM. As can be seen from Table 1, the diameter of $Al(OH)_3$ Mp measured by particle size analyzer ranged from 1.98-15.17 μm, with the average hydrodynamic diameter of 4.21 μm. On the other hand, the dominant diameter of $Al(OH)_3$ Np fell around 0.150 μm with the mean diameter of 0.162 μm and narrow size distribution (0.12-0.58μm), indicating the affectivity of high shear homogenization to reduce the particle size. In comparisons to these two samples, the average particle size of Alhydrogel® measured in this study was 4.02 μm with almost identical particle size range to those of $Al(OH)_3$ Mp.

From morphological observation (Fig. 1), the TEM image of $Al(OH)_3$ Np clearly showed the aggregate formation with the size of single nano structure was in the order of 30 nm. The particle size variance between TEM image and static light scattering measurement was due to the difference of principle used of these two apparatuses. Particle size analyzer measures the size distribution of the particle on aqueous suspension which may have high tendency of particle aggregation to take place. On the other hand, TEM image can directly measures the diameter of single particle and observes its morphological shape on dry state. The TEM photograph also revealed that $Al(OH)_3$ Np exhibited relatively uniform and spherical shape structure. Such similar morphological shape was also identified in the TEM image of $Al(OH)_3$ Mp (data not shown). Also, $Al(OH)_3$ Np had slight lower isoelectric point (PI)and pH compared to those of $Al(OH)_3$ Mp and Alhydrogel® (Table 1), which may attributed to the addition of stabilizer.

Acute toxicity: During the acute toxicity test, $Al(OH)_3$ samples at all doses did not induce any mortality effect to the treated mice (Table 2). Therefore, the LD_{50} of all type of $Al(OH)_3$ was estimated to be higher than 81.92 mg kg^{-1}. Following the sample administration, the morbidity of the animal such as

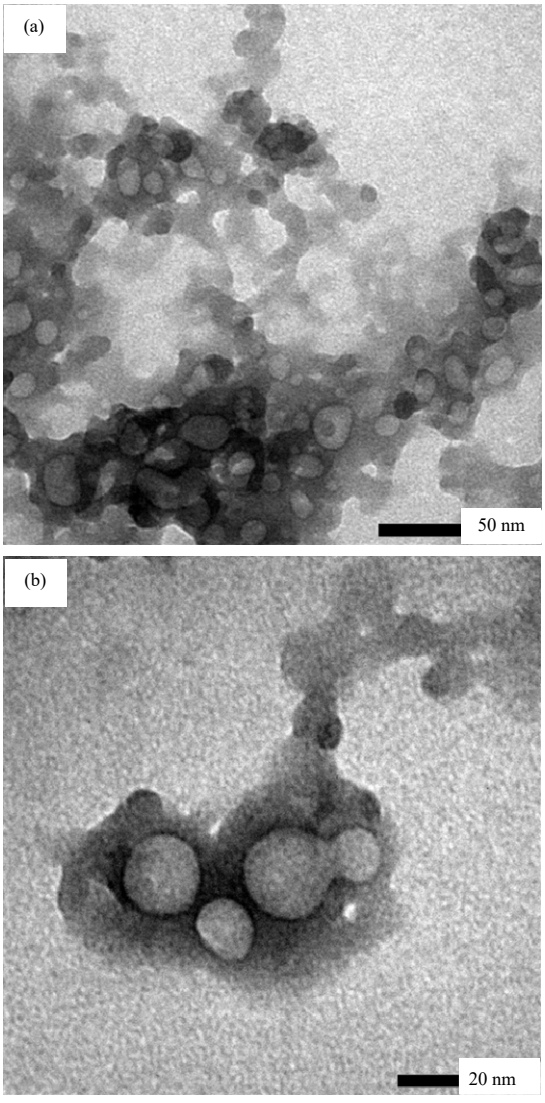

Fig. 1(a-b): (a-b) TEM image of $Al(OH)_3$ Np

Table 1: Characteristics of $Al(OH)_3$ used in this study

Samples	Al content (mg mL^{-1})	Average diameter (μm)[c]	Particle size range (μm)[c]	pH	Isoelectric point (PI)
Alhydrogel®	6.50[a]	4.02	1.73-15.17	6.33	8.18
$Al(OH)_3$ Mp	12.12[b]	4.21	1.98-15.17	7.46	9.20
$Al(OH)_3$ Np	13.39[b]	0.162	0.12-0.58	5.54	7.20

[a]Based on information written on the certificate analysis of the Alhydrogel®, [b]Measured by X-Ray fluorescence (XRF) spectrometers, [c]Measured by light scattering analysis

Table 2: Mortality data of test animal

Treatments	$Al(OH)_3$ dose (mg kg^{-1})	Incidence of death/number of animals
Phosphate-buffered saline	0.00	0/5
Alhydrogel®	0.16	0/5
	1.28	0/5
	10.24	0/5
	81.92	0/5
$Al(OH)_3$ Mp	0.16	0/5
	1.28	0/5
	10.24	0/5
	81.92	0/5
$Al(OH)_3$ Np	0.16	0/5
	1.28	0/5
	10.24	0/5
	81.92	0/5

Table 3: Behavioral and physical symptoms observation of test animal within 4 h of sample administration

		Symptoms (number of animal)			
Treatments	$Al(OH)_3$ dose (mg kg^{-1})	Tremor	Drowsiness	Hypoactivity	Piloerection
Phosphate-buffered saline	0.00	0/5	0/5	0/5	0/5
Alhydrogel®	0.16	0/5	3/5*	0/5	2/5
	1.28	1/5	0/5	0/5	1/5
	10.24	0/5	0/5	0/5	1/5
	81.92	0/5	5/5*	5/5*	2/5
$Al(OH)_3$ Mp	0.16	0/5	2/5	2/5*	2/5
	1.28	0/5	0/5	0/5	1/5
	10.24	1/5	4/5*	4/5*	2/5
	81.92	2/5	5/5*	5/5*	2/5
$Al(OH)_3$ Np	0.16	1/5	3/5	3/5	3/5
	1.28	0/5	2/5	2/5	1/5
	10.24	0/5	5/5*	5/5*	1/5
	81.92	1/5	5/5*	5/5*	3/5

Statistical significance was evaluated using one-way analysis of variance (ANOVA) (*$p<0.05$)

Table 4: Extended behavioral and physical symptoms observation of test animal after PBS administration

	Symptoms			
Days	Tremor	Drowsiness	Hypoactivity	Piloerection
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-
5	-	-	-	-
6	-	-	-	-
7	-	-	-	-
8	-	-	-	-
9	-	-	-	-
10	-	-	-	-
11	-	-	-	-
12	-	-	-	-
13	-	-	-	-
14	-	-	-	-

+: Symptom observed, -: Symptom was not observed

tremor, drowsiness, hypoactivity and piloerection was examined, as resumed in Table 3. At the 1st day of sample administration, only animal treated with the higher dose showed drowsiness and hypoactivity symptoms with statistically significant difference compared to those of control animal. In the next days, however, the drowsiness and hypoactivity effect was vanished (Table 4-7).

The body weights profile of the tested animals under observation period is shown in Table 8. The obtained result revealed that there was no difference in alteration of body weight of all sample-treated mice compared to those of control group throughout the study period. Although there was a slight attenuation on body weight profile at one day after sample administration which presumably due to the stress caused by sample administration, all mice resumed their body weight development in the next day. Thus, it may indicate that $Al(OH)_3$ has a negligible toxicity on the animal growth.

The postmortem observation of mice organ including liver, kidney, spleen, lung and hearth was also conducted (Table 8). Visually, there is no remarkable change of organ compared to those of control group. In organ index evaluation, $Al(OH)_3$administration did not alter the relative organ weights, except those of kidney and spleen of mice treated with $Al(OH)_3$ Np. The $Al(OH)_3$ Np at all doses induced a significant increase of kidney index. Similarly, $Al(OH)_3$ Np at doses of 1.28 and 10.24 mg kg^{-1} altered spleen index as compared to PBS-administered mice.

Table 5: Extended behavioral and physical symptoms observation of test animal after Alhydrogel® administration

	Symptoms															
	Tremor				Drowsiness				Hypoactivity				Piloerection			
	$Al(OH)_3$ dose (mg kg^{-1})				$Al(OH)_3$ dose (mg kg^{-1})				$Al(OH)_3$ dose (mg kg^{-1})				$Al(OH)_3$ dose (mg kg^{-1})			
Days	0.16	1.28	10.24	81.92	0.16	1.28	10.24	81.92	0.16	1.28	10.24	81.92	0.16	1.28	10.24	81.92
2	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+
3	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+
4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+: Symptom observed, -: Symptom was not observed

Table 6: Extended behavioral and physical symptoms observation of test animal after $Al(OH)_3$ Mp administration

	Symptoms															
	Tremor				Drowsiness				Hypoactivity				Piloerection			
	$Al(OH)_3$ dose (mg kg^{-1})				$Al(OH)_3$ dose (mg kg^{-1})				$Al(OH)_3$ dose (mg kg^{-1})				$Al(OH)_3$ dose (mg kg^{-1})			
Days	0.16	1.28	10.24	81.92	0.16	1.28	10.24	81.92	0.16	1.28	10.24	81.92	0.16	1.28	10.24	81.92
2	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
3	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-	+
4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+: Symptom observed, -: Symptom was not observed

The histological specimens of lung, liver, kidney and spleen are shown in Fig. 2-5. In the tissue section of lung (Fig. 2), thickening of alveolar septal walls and pulmonary arterioles dilatation were obviously found, especially in mice treated with the highest dose of $Al(OH)_3$. Also, granuloma and eosinophilic infiltration were identifiable in all groups which indicating lung inflammation. The histological photomicrographs of the liver are shown in Fig. 3. In general, normal hepatocytes arrangement forming trabecular pattern was observed in all tissue sections. Also, mild inflammation and necrosis were noticed in those of PBS-and sample-treated groups. The notable morphological change in liver histology of sample-treated mice appeared in the form of granuloma and lymphocytic infiltration, in which the tissue section from $Al(OH)_3$ Np treated mice showed more in ltrating mononuclear cells than those from the other groups. In addition, minor enlargement of hepatocyte and binucleated cells were observed occasionally, both those of control group and sample-treated groups. With regard to kidney histopathological study (Fig. 4), the tissue section of all sample-treated groups was normal, it exhibited preserved glomerular and tubular structure, relatively similar to those of the control group. Also, the H and E staining of splenic sections from control-and sample-treated groups displayed normal spleen architecture without any major histological abnormality, even at high concentrations of sample (Fig. 5).

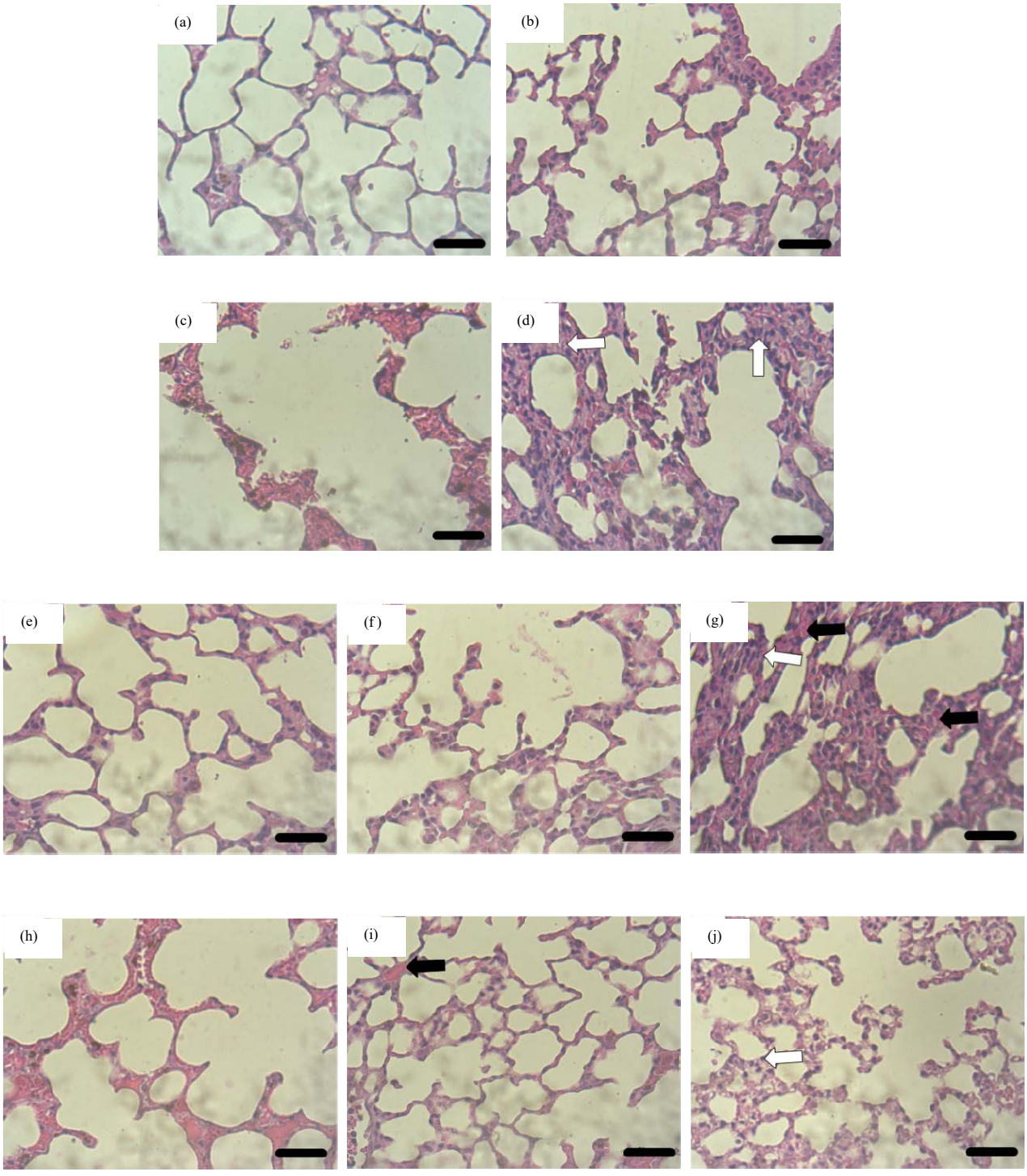

Fig. 2(a-j): Representative images of lung histopathology, (a) Control mice, (b-d) Alhydrogel® treated mice with administration dose of (b) 1.28 mg kg^{-1}, (c) 10.24 mg kg^{-1}, (d) 80.92 mg kg^{-1}, (e-g) $Al(OH)_3$ Mp treated mice with administration dose of (e) 1.28 mg kg^{-1}, (f) 10.24 mg kg^{-1} and (g) 80.92 mg kg^{-1}, (h-j) $Al(OH)_3$ Np treated mice with administration dose of (h) 1.28 mg kg^{-1}, (i) 10.24 mg kg^{-1} and (j) 80.92 mg kg^{-1}

White arrows show pulmonary interstitial thickening, black arrows show pulmonary arterioles dilatation and congestion, tissue section was stained with H and E and examined by light microscopy (40x magnification), scale bar 100 μm

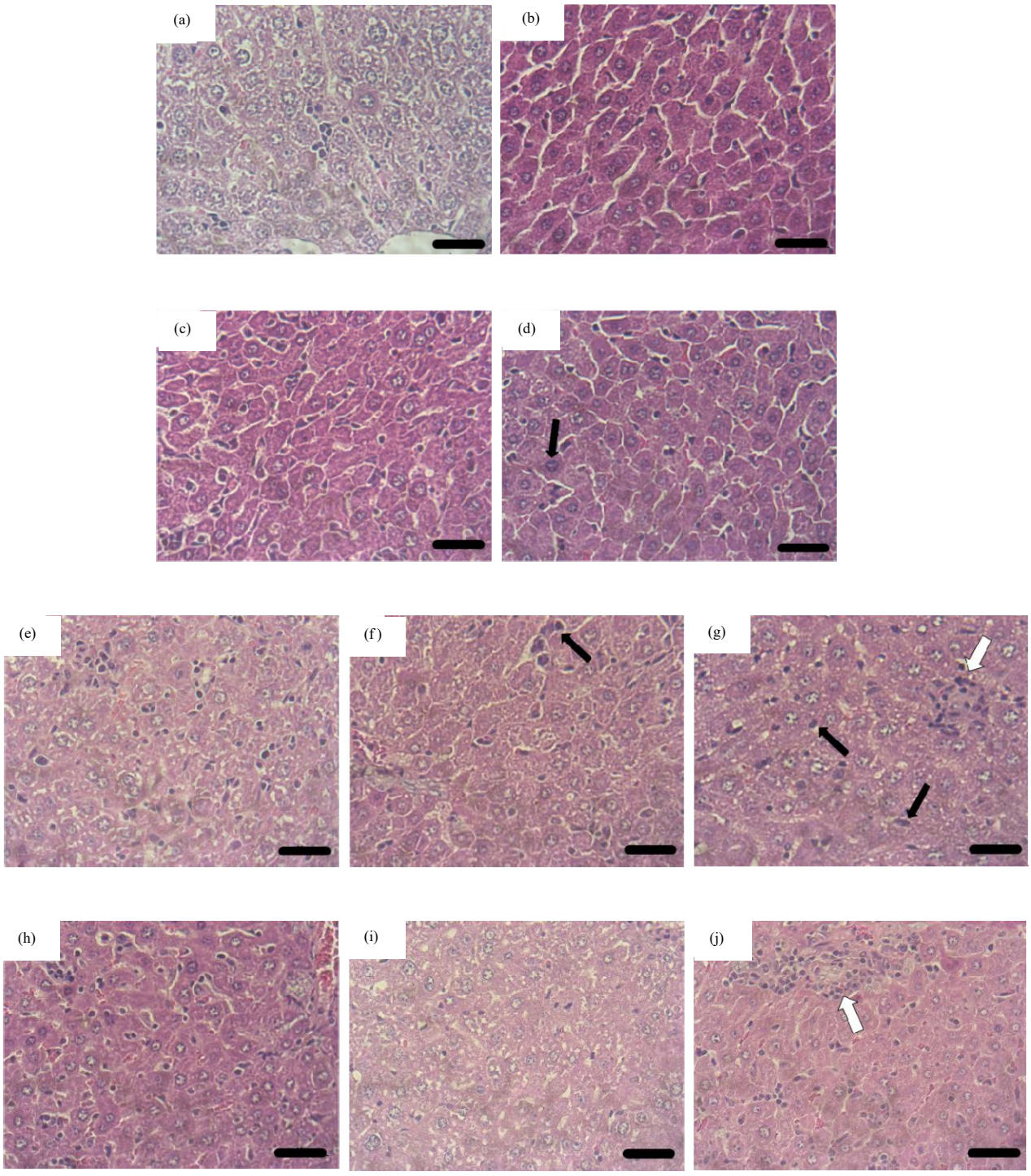

Fig. 3(a-j): Representative images of liver histopathology, (a) Control mice, (b-d) Alhydrogel® treated mice with administration dose of (b) 1.28 mg kg^{-1}, (c) 10.24 mg kg^{-1}, (d) 80.92 mg kg^{-1}, (e-g) $Al(OH)_3$ Mp treated mice with administration dose of (e) 1.28 mg kg^{-1}, (f) 10.24 mg kg^{-1}, (g) 80.92 mg kg^{-1}, (h-j) $Al(OH)_3$ Np treated mice with administration dose of (h) 1.28 mg kg^{-1}, (i) 10.24 mg kg^{-1} and (j) 80.92 mg kg^{-1}

White arrows show lymphocytic and eosinophilic infiltrates, black arrows show hepatic cell swelling, tissue section was stained with H and E and examined by light microscopy (40x magnification), scale bar 100 µm

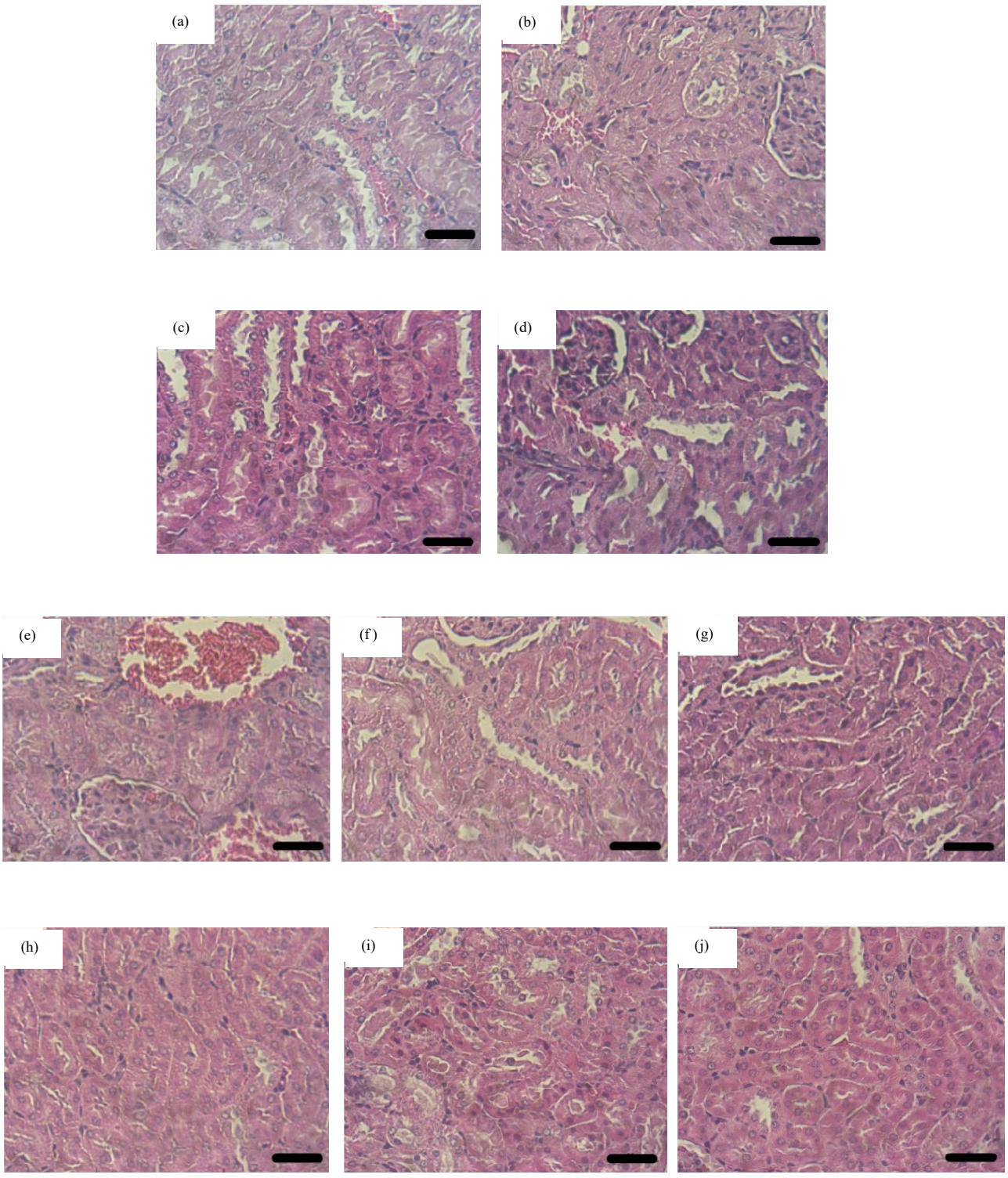

Fig. 4(a-j): Representative images of kidney histopathology, (a) Control mice, (b-d) Alhydrogel® treated mice with administration dose of (b) 1.28 mg kg^{-1}, (c) 10.24 mg kg^{-1}, (d) 80.92 mg kg^{-1}, (e-g) $Al(OH)_3$ Mp treated mice with administration dose of (e) 1.28 mg kg^{-1}, (f) 10.24 mg kg^{-1}, (g) 80.92 mg kg^{-1}, (h-j) $Al(OH)_3$ Np treated mice with administration dose of (h) 1.28 mg kg^{-1}, (i) 10.24 mg kg^{-1} and (j) 80.92 mg kg^{-1}

Tissue section was stained with H and E and examined by light microscopy (40x magnification), scale bar 100 μm

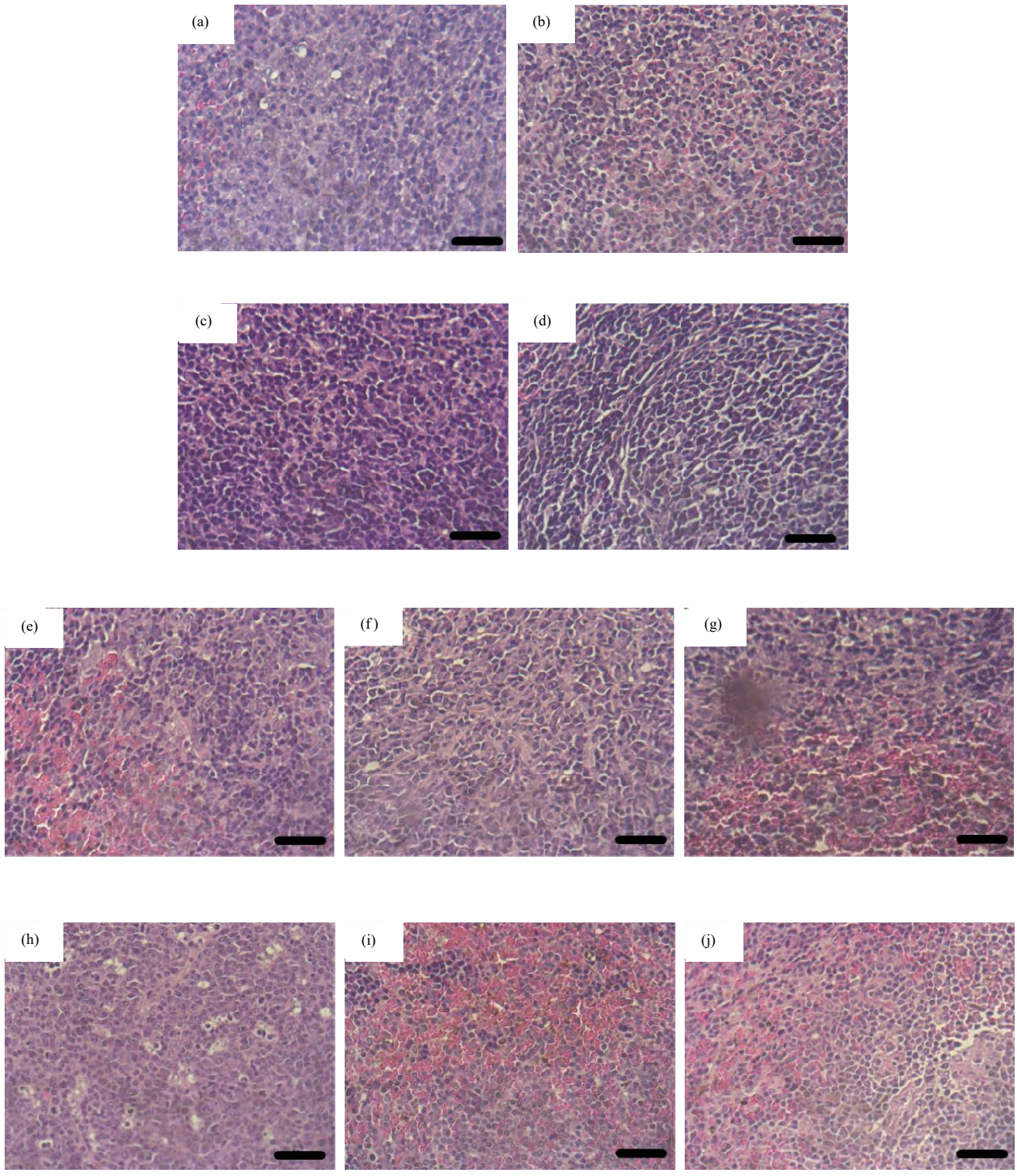

Fig. 5(a-j): Representative images of spleen histopathology, (a) Control mice, (b-d) Alhydrogel® treated mice with administration dose of (b) 1.28 mg kg^{-1}, (c) 10.24 mg kg^{-1}, (d) 80.92 mg kg^{-1}, (e-g) $Al(OH)_3$ Mp treated mice with administration dose of (e) 1.28 mg kg^{-1}, (f) 10.24 mg kg^{-1}, (g) 80.92 mg kg^{-1}, (h-j) $Al(OH)_3$ Np treated mice with administration dose of (h) 1.28 mg kg^{-1}, (i) 10.24 mg kg^{-1} and (j) 80.92 mg kg^{-1}

Tissue section was stained with H and E and examined by light microscopy (40x magnification).N 5, scale bar 100 µm

Table 7: Extended behavioral and physical symptoms observation of test animal after $Al(OH)_3$ Np administration

	Symptoms															
	Tremor				Drowsiness				Hypoactivity				Piloerection			
	$Al(OH)_3$ dose (mg kg^{-1})				$Al(OH)_3$ dose (mg kg^{-1})				$Al(OH)_3$ dose (mg kg^{-1})				$Al(OH)_3$ dose (mg kg^{-1})			
Days	0.16	1.28	10.24	81.92	0.16	1.28	10.24	81.92	0.16	1.28	10.24	81.92	0.16	1.28	10.24	81.92
2	-	-	-	-	-	-	-	-	+	-	+	+	+	-	+	+
3	-	-	-	-	-	-	-	-	+	-	-	+	+	+	+	+
4	-	-	-	-	+	-	-	-	+	-	-	-	-	+	+	+
5	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
7	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+
8	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+
9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+: Symptom observed, -: Symptom was not observed

Table 8: Body and organ weight of test animal

		Body weight (g)		Organ weight/mice body weight (%)				
Treatments	$Al(OH)_3$ dose (mg kg^{-1})	Before	After	Liver	Kidney	Spleen	Lungs	Heart
PBS	0.00	19.6±2.1	27.0±3.2	5.8±0.8	1.1±0.1	0.8±0.1	0.8±0.3	0.5±0.1
Alhydrogel®	0.16	19.6±1.1	23.6±2.2	7.5±1.2	1.2±0.3	1.3±0.9	0.9±0.3	0.5±0.1
	1.28	19.8±4.0	25.0±2.2	4.9±1.1	1.1±0.1	0.8±0.7	0.7±0.2	0.5±0.1
	10.24	20.2±2.1	26.3±3.6	5.6±0.6	1.0±0.1	1.0±0.5	0.7±0.5	0.4±0.1
	81.92	20.2±2.7	25.7±3.3	5.8±1.6	1.2±0.5	0.9±0.4	0.8±0.4	0.3±0.1
$Al(OH)_3$ Mp	0.16	22.2±1.7	28.2±3.2	6.2±1.1	1.2±0.2	0.9±0.3	0.6±0.2	0.4±0.1
	1.28	20.1±2.5	26.3±2.9	5.9±1.0	1.0±0.1	1.1±0.4	0.7±0.3	0.4±0.1
	10.24	21.6±3.5	28.2±2.1	5.5±0.9	1.2±0.2	0.8±0.4	0.7±0.1	0.5±0.1
	81.92	22.2±2.7	27.1±1.3	5.8±0.5	1.0±0.1	1.0±0.3	0.6±0.1	0.3±0.1
$Al(OH)_3$ Np	0.16	19.5±3.1	22.9±1.5	6.6±1.7	1.7±0.5*	1.1±0.3	1.2±0.6	0.5±0.1
	1.28	20.2±2.0	27.0±2.4	7.9±2.0	2.0±0.3*	1.5±0.4*	1.5±0.4	0.6±0.1
	10.24	19.5±2.3	24.0±2.5	8.3±1.3	1.8±0.2*	1.5±0.6*	1.4±0.5	0.6±0.1
	81.92	21.0±2.1	27.4±3.3	7.0±1.7	1.3±0.4*	1.1±0.5	1.2±0.4	0.4±0.1

Statistical significance was evaluated using one-way analysis of variance (ANOVA) (*$p<0.05$), PBS: Phosphate-buffered saline

DISCUSSION

In the present study, $Al(OH)_3$ Np with sub-micrometer size was successfully produced and importantly, its intraperitoneal acute toxicity profile was evaluated. The synthesized $Al(OH)_3$ Np was not only demonstrated superior adjuvant effect compared to its micro-sized counterpart and commercial $Al(OH)_3$ as was proved in the previous studies by Pambudi *et al.*[16] and Sun *et al.*[25], but also exerted a relatively low intraperitoneal acute toxicity. This toxicity profile is a key issue in the development of future nano-sized $Al(OH)_3$ adjuvant.

The application of down-sizing strategy on alum-based adjuvant as we did in this research is a popular alternative to further improve its immunostimulant activity and could contribute to the next-generation vaccine development. To generate $Al(OH)_3$ Nps, various top-down manufacturing method can be used, such as high through high-shear homogenization, ultrasonication and high-pressure microfluidization[14,15,26]. These strategies have been reported could successfully produce nanoscale $Al(OH)_3$ with the average diameter of less than 200 nm and importantly increase the immune response[13,25]. Considering the scaling-up production process, we developed simple fabrication method to produce $Al(OH)_3$ NPs by high-shear homogenization. For Alhydrogel®, it was known from previous study that its stock suspension has a relatively big median size (2.67 μm) and large polydispersity index[11,26]. It displayed a broad and heterogeneous particle size distribution, ranging from 0.5-10 μm, due to it highly tendency to form an aggregate[14].

The prepared $Al(OH)_3$ Nps were stabilized by the addition of low-concentration of sodium polyphosphate, resulting in a stable particle size over 2 months when stored at room temperature (data not shown). From our previous study, sodium polyphosphate seems to be most appropriate stabilizer compared to those of trehalose and polyvinyl alcohol[27]. Also, in drug delivery research, sodium polyphosphate has been intensively used as a crosslink agent as well as a stabilizer for metal oxide nanoparticulate[28-30]. In contrast, Alhydrogel® and $Al(OH)_3$ Mps without any addition of stabilizer easily formed aggregate during the storage due to the flocculation, yet this micro-suspension could be quickly re-dispersed using physical agitation. However, It worth to note that the addition of sodium polyphosphate in $Al(OH)_3$ Np suspension should be controlled. The excess of phosphate ion may alter the physicochemical character of $Al(OH)_3$ NPs as the ligand exchange adsorption of the phosphate ion with the hydroxyl group of $Al(OH)_3$ could take place[31]. This phenomena could be observed from the reduced PI value of $Al(OH)_3$ Np. The exposure of phosphate ion from the sodium polyphosphate reduced PI value of $Al(OH)_3$ Np to be around 7.2, while those of Alhydrogel® and $Al(OH)_3$ Mp was 8.18 and 9.22, respectively. This PI alteration was consistent with previous research that reporting the reduced PI of $Al(OH)_3$ into 4.0 due to the substitution of phosphate for hydroxyl[32]. For some antigens, the PI change due to the ligand exchange may critically affect protein binding capacity[33-35] of the $Al(OH)_3$. In morphological evaluation using TEM, $Al(OH)_3$ Np exhibited spherical structure. Meanwhile, it was known that the common $Al(OH)_3$ used for adjuvant including Alhydrogel® displayed fiber-likemicro-crystalline substructure. Such structural variations may be caused by the different preparation procedure and the chemical precursor used for producing[36] $Al(OH)_3$.

Besides highlighting the synthesis and physicochemical characterizations of $Al(OH)_3$, the principal purpose in this research was to elucidate the toxicity profile of the nanosized $Al(OH)_3$ as this toxicity study nano-scale $Al(OH)_3$ has been characterized to a much lesser degree. It was well-known that engineered nanomaterials may have higher toxicity compared those in bulk form which may lead to undesirable effects. For example, the LD50 of bulk ZnO in mice after intratracheal instillation was 7950 mg kg^{-1}, while ZnO nanoparticle having diameter around 20 nm had increased toxicity[36] with LD_{50} of 5 mg kg^{-1}. In another study, the LD_{50} of 20 and 50 nm silver nanoparticles in mice were estimated at 169 and 354 mg kg^{-1}, indicating the critical effect of particle size on toxicity profile of nanomaterial[37]. For aluminum-based material, the toxicity is highly dependent on its salt form. In general, the acute toxicity of aluminum compounds was relatively low due to the low solubility of these compounds[38]. The LD_{50} of bulk form of $Al(OH)_3$ on rat through oral administration was higher than 2000 mg kg^{-1} as only less than 0.01% $Al(OH)_3$ was absorbed into the blood stream[39]. Lack of toxicity sign of $Al(OH)_3$ in human following acute administration was also demonstrated[40]. In this study, dose of tested materials up to than 81.92 mg kg^{-1} was clinically safe and did not cause any lethal effect on mice. This finding seems consistent with the previous work that reported no lethal sign on Sprague-Dawley rats after oral administration of higher dose of $Al(OH)_3$ (302 mg kg^{-1}/day) for 28 days[41]. For the future work, sample with higher dose of $Al(OH)_3$ are needed to obtain exact LD_{50}, especially for those of $Al(OH)_3$ Np.

Although the administered samples in this study did not display any lethal effect to the mice, some toxicological examinations, including behavioral alteration, body weight growth, organ index and organ histopathology analysis were carried out to comprehensively observe any undesired effect of $Al(OH)_3$. In this study, the clinical symptoms shared among groups that took place during the sample administration day were drowsiness and hypoactivity and importantly such effects did not remain longer in mice. From body weight profile, all the mice showed positive trend after 14 day-post sample injection, the increase of body weight ranged from 21-35%. It was known that the 10% decrease of body weight from its initial value indicated the occurrence of side effect of the tested material as a body weight is an important marker of gross toxicity[42]. Hence, the administered samples in present research emphasized a non-toxic character of the tested $Al(OH)_3$. From organ mass index data, mice treated with $Al(OH)_3$ Np increased kidney and spleen mass coefficient which may attribute to the inflammation on these organs. However, the alteration of organ mass index did not necessarily result in change of tissue architecture; the histological examination of kidney and spleen treated with $Al(OH)_3$ Np displayed no significant difference compared to those of control group. It could reveal that spleen and kidney may be the main organ which are responsible for filtering and clearing the accumulation of foreign particle of $Al(OH)_3$ Np. Such spleen alteration may be facilitated by macrophage as was observed by Kwon *et al.*[43] when observing the biodistribution of inhaled fluorescent magnetic nanoparticles. The macrophage activation in spleen, however, did not cause histological accumulation of megakaryocytes in the red pulp as the spleen microstructure was normal and no pathological change was observed. Although sample was administered through intraperitoneal route, histology of lung tissue-treated with the highest dose of samples displayed noticeable effect. It thus may indicate the absorption of administered sample into systemic circulation before reaching lung.

Tremendous effort has been spent on developing of nanoparticle of aluminum salts adjuvant[12,25,44-46] as well as an attempt to understand the exact immunostimulating mechanism underlying their strong adjuvant activity[47]. However, the *in vivo* toxicity study of $Al(OH)_3$ Nps, to the best of our knowledge, has not been performed yet. Despite the favorable safety profile of $Al(OH)_3$ in its conventional bulk form, the toxicity of $Al(OH)_3$ NPs as a different entity is an important factor that should be investigated to answer the questioned biosafety of nano materials. Therefore, this study provided knowledge of toxicity profile of $Al(OH)_3$ Nps administered through intraperitoneal administration, that could imply its safety for further development of nano-range particulates adjuvant.

CONCLUSION

Considering all findings in this study, it can be inferred that the toxicity profile of $Al(OH)_3$ Np at the highest dose used in this research was not significantly different compared to those of control, Alhydrogel® and $Al(OH)_3$ Mp-treated group. No permanent behavioral alterations were induced during 14-day observation period. To complete the histological data, further organ-related biochemical analysis and measurement of $Al(OH)_3$ accumulation in major organs should be carried out in the future for probing any abnormality in the organ.

SIGNIFICANCE STATEMENT

The findings of this study describes the intraperitoneal acute toxicity profile of $Al(OH)_3$ Np. Although further toxicological assays are needed to complete this study, the current data indicated the low intraperitoneal toxicity of $Al(OH)_3$ Np as its obtained data was not significantly different compared to those of control counterparts. Thus, it will help the researcher to uncover the high potency of $Al(OH)_3$ Np and can be beneficial for further development of a new generation vaccine adjuvant.

ACKNOWLEDGMENT

This study is supported by Research Grant from Ministry of Science, Technology and Higher Education of Republic Indonesia (INSINAS 2019).

REFERENCES

1. Glenny, A.T., C.G. Pope, H. Waddington and U. Wallace, 1926. Immunological notes. XVII-XXIV. J. Pathol. Bacteriol., 29: 31-40.
2. Gupta, R.K., B.E. Rost, E. Relyveld and G.R. Siber, 1995. Adjuvant Properties of Aluminum and Calcium Compounds. In: Vaccine Design: The Subunit and Adjuvant Approach, Powell, M.F. and M.J. Newman (Eds.). Springer, Boston, MA., USA., ISBN: 978-1-4615-1823-5, pp: 229-248.
3. Gupta, R.K., 1998. Aluminum compounds as vaccine adjuvants. Adv. Drug Deliv. Rev., 32: 155-172.
4. O'Hagan, D.T., M.L. MacKichan and M. Singh, 2001. Recent developments in adjuvants for vaccines against infectious diseases. Biomol. Eng., 18: 69-85.
5. Noe, S.M., M.A. Green, H. HogenEsch and S.L. Hem, 2010. Mechanism of immunopotentiation by aluminum-containing adjuvants elucidated by the relationship between antigen retention at the inoculation site and the immune response. Vaccine, 28: 3588-3594.
6. He, P., Y. Zou and Z. Hu, 2015. Advances in aluminum hydroxide-based adjuvant research and its mechanism. Hum. Vaccines Immunotherapeut., 11: 477-488.
7. Exley, C., P. Siesjo and H. Eriksson, 2010. The immunobiology of aluminium adjuvants: How do they really work? Trends Immunol., 31: 103-109.
8. Hutchison, S., R.A. Benson, V.B. Gibson, A.H. Pollock, P. Garside and J.M. Brewer, 2012. Antigen depot is not required for alum adjuvanticity. FASEB J., 26: 1272-1279.
9. Willhite, C.C., N.A. Karyakina, R.A. Yokel, N. Yenugadhati and T.M. Wisniewski *et al*., 2014. Systematic review of potential health risks posed by pharmaceutical, occupational and consumer exposures to metallic and nanoscale aluminum, aluminum oxides, aluminum hydroxide and its soluble salts. Crit. Rev. Toxicol., 44: 1-80.
10. Morefield, G.L., A. Sokolovska, D. Jiang, H. HogenEsch, J.P. Robinson and S.L. Hem, 2005. Role of aluminum-containing adjuvants in antigen internalization by dendritic cells *in vitro*. Vaccine, 23: 1588-1595.
11. Shardlow, E., M. Mold and C. Exley, 2017. From stock bottle to vaccine: Elucidating the particle size distributions of aluminum adjuvants using dynamic light scattering. Front. Chem., Vol. 4. 10.3389/fchem.2016.00048
12. Sun, B., Z. Ji, Y.P. Liao, C.H. Chang and X. Wang *et al*., 2017. Enhanced Immune adjuvant activity of aluminum oxyhydroxide nanorods through cationic surface functionalization. ACS Applied Mater. Interfaces, 9: 21697-21705.
13. Dong, H., Z.F. Wen, L. Chen, N. Zhou and H. Liu *et al*., 2018. Polyethyleneimine modification of aluminum hydroxide nanoparticle enhances antigen transportation and cross-presentation of dendritic cells. Int. J. Nanomed., 13: 3353-3365.
14. Orr, M.T., A.P. Khandhar, E. Seydoux, H. Liang and E. Gage *et al*., 2019. Reprogramming the adjuvant properties of aluminum oxyhydroxide with nanoparticle technology. npj Vaccines, Vol. 4. 10.1038/s41541-018-0094-0.

15. Mardliyati, E., D.R. Setyawati, S. Pambudi, Suryandaru, R. Kusumaningrum and M.I. Amal, 2017. Preparation of aluminum hydroxide by precipitation method for vaccine adjuvant application. Int. J. Eng. Res. Applic., 7: 21-25.
16. Pambudi, S., E. Mardliyati, S. Rahmani, D.R. Setyawati and T. Widayanti *et al*., 2018. The potency of aluminum hydroxide nanoparticles for dengue subunit vaccine adjuvant. Microbiol. Indonesia, 12: 99-105.
17. MacMicking, J., Q.W. Xie and C. Nathan, 1997. Nitric oxide and macrophage function. Annu. Rev. Immunol., 15: 323-350.
18. Bosca, L., M. Zeini, P.G. Traves and S. Hortelano, 2005. Nitric oxide and cell viability in inflammatory cells: A role for NO in macrophage function and fate. Toxicology, 208: 249-258.
19. Lin, Y.F., M.C. Deng, L.P. Tseng, P.R. Jiang, T.R. Jan, F.I. Hsieh and D.Z. Liu, 2011. Adjuvant effect of liposome in chicken result from induction of nitric oxide. Biomed. Mater., Vol. 6, No. 1. 10.1088/1748-6041/6/1/015011.
20. Gherardi, R.K., J. Aouizerate, J. Cadusseau, S. Yara and F.J. Authier, 2016. Aluminum adjuvants of vaccines injected into the muscle: Normal fate, pathology and associated disease. Morphologie, 100: 85-94.
21. Kelso, J.M., M.J. Greenhawt, J.T. Li, R.A. Nicklas and D.I. Bernstein *et al*., 2012. Adverse reactions to vaccines practice parameter 2012 update. J. Allergy Clin. Immunol., 130: 25-43.
22. Tubaro, A., S. Sosa, M. Carbonatto, G. Altinier and F. Vita *et al*., 2003. Oral and intraperitoneal acute toxicity studies of yessotoxin and homoyessotoxins in mice. Toxicon, 41: 783-792.
23. HogenEsch, H., D.T. O'Hagan and C.B. Fox, 2018. Optimizing the utilization of aluminum adjuvants in vaccines: You might just get what you want. npj Vaccines, Vol. 3. 10.1038/s41541-018-0089-x.
24. Slaoui, M. and L. Fiette, 2011. Histopathology Procedures: From Tissue Sampling to Histopathological Evaluation. In: Drug Safety Evaluation: Methods and Protocols, Gautier, J.C. (Ed.). Humana Press, Totowa, NJ., USA., ISBN: 978-1-60761-849-2, pp: 69-82.
25. Sun, B., Z. Ji, Y.P. Liao, M. Wang and X. Wang *et al*., 2013. Engineering an effective immune adjuvant by designed control of shape and crystallinity of aluminum oxyhydroxide nanoparticles. ACS Nano, 7: 10834-10849.
26. Harris, J.R., A. Soliakov, R.J. Lewis, F. Depoix, A. Watkinson and J.H. Lakey, 2012. Alhydrogel® adjuvant, ultrasonic dispersion and protein binding: A TEM and analytical study. Micron, 43: 192-200.
27. Mardliyati, E., S. Rahmani, S. El Muttaqien and D.R. Setyawati, 2019. Effect of stabilizers on physical stability and protein adsorptive capacity of aluminum hydroxide nanoparticles suspension. Int. J. Eng. Res. Applic., 9: 34-37.
28. Gao, Y.X., J.W. Xin, Z.Y. Shen, W. Pan, X. Li and A.G. Wu, 2013. A new rapid colorimetric detection method of Mn^{2+} based on tripolyphosphate modified silver nanoparticles. Sens. Actuat. B: Chem., 181: 288-293.
29. Zamora-Mora, V., M. Fernandez-Gutierrez, J. San Roman, G. Goya, R. Hernandez and C. Mijangos, 2014. Magnetic core-shell chitosan nanoparticles: Rheological characterization and hyperthermia application. Carbohydr. Polym., 102: 691-698.
30. Kujda, M., M. Ocwieja, Z. Adamczyk, O. Bochenska and G. Bras *et al*., 2015. Charge stabilized silver nanoparticles applied as antibacterial agents. J. Nanosci. Nanotechnol., 15: 3574-3583.
31. Hem, S.L. and C.T. Johnston, 2014. Production and Characterization of Aluminum-Containing Adjuvants. In: Vaccine Development and Manufacturing, Wen, E.P., R. Ellis and N.S. Pujar (Eds.). Chapter 11, John Wiley & Sons, Hoboken, NJ., USA., ISBN: 9780470261941, pp: 319-346.
32. Iyer, S., H. HogenEsch and S.L. Hem, 2003. Effect of the degree of phosphate substitution in aluminum hydroxide adjuvant on the adsorption of phosphorylated proteins. Pharmaceut. Dev. Technol., 8: 81-86.
33. Jiang, D., C.T. Johnston and S.L. Hem, 2003. Using rate of acid neutralization to characterize aluminum phosphate adjuvant. Pharmaceut. Dev. Technol., 8: 349-356.
34. Hem, S.L. and H. HogenEsch, 2007. Relationship between physical and chemical properties of aluminum-containing adjuvants and immunopotentiation. Expert Rev. Vaccines, 6: 685-698.
35. Morefield, G.L., D. Jiang, I.Z. Romero-Mendez, R.L. Geahlen, H. HogenEsch and S.L. Hem, 2005. Effect of phosphorylation of ovalbumin on adsorption by aluminum-containing adjuvants and elution upon exposure to interstitial fluid. Vaccine, 23: 1502-1506.
36. Wang, D., H. Li, Z. Liu, J. Zhou and T. Zhang, 2017. Acute toxicological effects of zinc oxide nanoparticles in mice after intratracheal instillation. Int. J. Occup. Environ. Health, 23: 11-19.
37. Elkhawass, E.A., M.E. Mohallal and M.F. Soliman, 2015. Acute toxicity of different sizes of silver nanoparticles intraperitonally injected in Balb/C mice using two toxicological methods. Int. J. Pharm. Pharmaceut. Sci., 7: 94-99.
38. Taiwo, O.A. and B. Storey-Laubach, 2001. Aluminum. In: Patty's Toxicology, Bingham, E., B. Cohrssen and C.H. Powell (Eds.). 5th Edn., John Wiley & Sons, Hoboken, NJ., USA., ISBN-13: 9780471319436, pp: 229-256.
39. Ingerman, L., L. Chappell, D.G. Jones, S. Keith and Z.A. Rosemond, 2008. Toxicological profile for aluminum. U.S. Department of Health and Human Services, Agency for Toxic Substances and Disease Registry, Atlanta, GA., USA., September 2008.

40. Wada, K., 1985. Aluminum. In: Metal and Human: Ecotoxicology and Clinical Medicine, Wada, K. (Ed.). Asakura Shoten, Tokyo, Japan, pp: 217-229.
41. Hicks, J.S., D.S. Hackett and G.L. Sprague, 1987. Toxicity and aluminium concentration in bone following dietary administration of two sodium aluminium phosphate formulations in rats. Food Chem. Toxicol., 25: 533-538.
42. Raza, M., O.A. Al-Shabanah, T.M. El-Hadiyah and A.A. Al-Majed, 2002. Effect of prolonged vigabatrin treatment on hematological and biochemical parameters in plasma, liver and kidney of Swiss albino mice. Sci. Pharmacuet., 70: 135-145.
43. Kwon, J.T., D.S. Kim, A. Minai-Tehrani, S.K. Hwang and S.H. Chang *et al*., 2009. Inhaled fluorescent magnetic nanoparticles induced extramedullary hematopoiesis in the spleen of mice. J. Occup. Health, 51: 423-431.
44. Li, X., S. Hufnagel, H. Xu, S.A. Valdes, S.G. Thakkar, Z. Cui and H. Celio, 2017. Aluminum (oxy)hydroxide nanosticks synthesized in bicontinuous reverse microemulsion have potent vaccine adjuvant activity. ACS Applied Mater. Interfaces, 9: 22893-22901.
45. Li, X., A.M. Aldayel and Z. Cui, 2014. Aluminum hydroxide nanoparticles show a stronger vaccine adjuvant activity than traditional aluminum hydroxide microparticles. J. Controlled Release, 173: 148-157.
46. Bilyy, R., S. Paryzhak, K. Turcheniuk, T. Dumych and A. Barras *et al*., 2019. Aluminum oxide nanowires as safe and effective adjuvants for next-generation vaccines. Mater. Today, 22: 58-66.
47. Ruwona, T.B., H. Xu, X. Li, A.N. Taylor, Y.C. Shi and Z. Cui, 2016. Toward understanding the mechanism underlying the strong adjuvant activity of aluminum salt nanoparticles. Vaccine, 34: 3059-3067.