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Original Article

Evaluation of the Toxic Effects of Aluminum Hydroxide Nanoparticles as Adjuvants in Vaccinated Neonatal Mice

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Abstract

Aluminum hydroxide nanoparticles have been employed in many industries, which are widely abundant in many aspects of human life. The role of the aluminum hydroxide nanoparticles adjuvant is to enhance the immune response. However, the impact of nanoparticles exposure has not been perfectly investigated yet. Accordingly, some questions have been raised about their potentially harmful effects, based on which the current research aims to answer them. This study aimed to investigate the histological effects of aluminum hydroxide nanoparticles and bulk-aluminum hydroxide (bulk $Al[OH]_3$) on the liver, lung, heart, and kidney tissues. For this reason, an experiment was implemented on the aluminum hydroxide nanoparticles adjuvant in five neonatal mice. Intramuscularly, the mice were injected with 0.125 mL of adjuvanted vaccine, while five neonatal mice were injected with bulk and nanoparticles of $Al(OH)_3$ and then sacrificed after one and two months, respectively. Vaccines were controlled by evaluating the histopathological response in neonatal mice. Subsequently, the pathological effect of both adjuvants was surveyed using the histological study of the lung, liver, heart, and kidney of the animals. The obtained recorded data indicated that both types of vaccine adjuvants caused pathological lesions on the histology sections of the liver, lung, heart, and kidney tissues. Moreover, bulk $Al(OH)_3$ adjuvant vaccine was more effective and had a higher pathological response than aluminum hydroxide nanoparticles adjuvant vaccine. In addition, the total DNA content in both groups was estimated using Fluorometer from Promega. Compared to aluminum hydroxide nanoparticles groups, the tissues indicated a decrease in total DNA content obtained in bulk $Al(OH)_3$ groups. Therefore, it can be concluded that the exposure to aluminum hydroxide nanoparticles would result in less pronounced toxicity, as well as systemic inflammation, compared to the bulk $Al(OH)_3$ aluminum hydroxide.

Keywords: $Al(OH)_3$NPs, Histopathology, Mice

1. Introduction

Nanotechnology is well known as one of the important and practical branches of modern science. It develops and manipulates some compounds to generate molecules via novel properties called nanoparticles (NPs) including particle size ranging from 1 to 100 nm (1). In this regard, the toxic effect of NPs can modulate various cellular and molecular components, resulting in altered tissue architecture. It is worthwhile to mention that at the molecular level, the toxicity of NPs could be directed on either structure or function of proteins, which may genetically change the certain protein-encoding (2). Physiologically, NPs affect different systems (3), such as the respiratory and reproductive systems (4).

In this regard, the aluminum (alum)-based adjuvants encounter several drawbacks, such as severe irritation and long duration inflammatory reaction of local tissues

(injection site), minimally induced cell-mediated, and immunity with a tendency to activate unfavorable uninvited immunoglobulin E (IgE)-mediated responses (4, 5). Accordingly, new adjuvants have been progressing to improve the immune response to weak antigens.

It should be mentioned that the nanomaterial contains unique physicochemical properties that differ from bulk materials of the same composition. In other words, it encompasses ultra-small size with a large surface area to mass ratio, as well as high reactivity. Likewise, such properties can be utilized to resolve some of the restrictions found in typical vaccines (6). Based on what was discussed above, this study aimed to explore the effects of alum and alum NPs applied as vaccine adjuvants on the histological structure of the liver, lung, heart, and kidney of neonatal immunized mice, as well as the concentration of DNA in these tissues.

2. Materials and Methods

2.1. Animals

To accomplish the aforementioned aim, 10 neonatal mice were used in an experiment. It should be noted that all animals were housed under standard laboratory conditions (i.e. the room temperature: 21.00±2.00˚C; relative humidity: 40.00%-55.00%; photoperiod: 12L:12D) in Macrolon cages. Meanwhile, all animals were fed via a standard breeding diet and tap water *ad libitum*.

2.2. Treatment

The alum-NPs nanopowder, with particle size of about 50 nm, was purchased from Sigma Aldrich® (CAS Number: 1344-28-1). In addition, the alum hydroxide was given as aqueous suspension (70 mg/kg BW), and then was chosen based on the study conducted by Park, Kim (7).

2.3. Extraction of DNA from the Tissue

As detailed in the extraction protocol provided by the supplier, DNA extraction was carried out using Qiagen® kit. To do so, 180 µl of buffer ATL were added to an Eppendorf tube containing 25 mg of tissues, followed by the addition of 20 µl of proteinase K in order to obtain the tissue lysate. Afterward, 200 µl of buffer ATL were added to all tubes, and then the tissue mixture was mixed thoroughly by vortexing. After that, 200 µl of ethanol (70%) was added to each tube, mixed, and vortexed thoroughly where the tubes were centrifuged (8000 rpm for 1 min). Moreover, another type of buffer (buffer AW1) was added (500 µl), followed by centrifugation (8000 rpm for 1 min). Subsequently, buffer AW1 was removed, and then buffer AW2 (500 µl) was added in which the tubes were centrifuged (14,000 rpm for 3 min) in order to dry the DNeasy membrane. Furthermore, the collection of DNA was carried out by adding 200 µl of buffer AE and pipetting onto the DNeasy membrane. The membranes were incubated at room temperature for 1 min, followed by centrifugation for 1 min at 8000 rpm for the DNA to elute.

2.4. Histological Study

In the following, some small specimens from the liver, lung, heart, and kidney were collected from the dissected mice and were fixed in neutral buffered formalin. Following that, 5µm sections were obtained to be stained via hematoxylin, and eosin was investigated using a light microscope (Leica DMLS) to identify the histological changes (8).

3. Results

3.1. Histological Study

The achieved results revealed that the use of either alum or NPs could cause severe histological changes in the liver. It should be mentioned that these changes included a wide range of pathological processes, such as mononuclear cell infiltration, hepatocellular necrosis, hydropic degeneration, and distinct Küpffer cells (Figure 1A, 1B). Furthermore, the use of alum adjuvant was severe in both groups of the histological changes of the kidney, resulting in mononuclear cell infiltration, vacuolation of renal tubule epithelial, and congestion of blood capillaries. Additionally, the observed histological changes were significant in the case of alum adjuvant and were more apparent at week 8 after immunization. Moreover, cellular necrosis and

inflammation in hepatocytes were observed in mice treated with Al $(OH)_3$ NPs associated with a vacuolated cell. Interestingly, as illustrated in Figure 1B, such improvements were more prevalent in the Al $(OH)_3$ NPs-treated mice. The histopathological changes in the liver, kidney, heart, and lung are exhibited in figures 1A, 1B; 2A, 2B; 3A, 3B; and 4A, 4B, respectively.

More importantly, the renal damage was observed in the kidney tissues treated with $Al(OH)_3$ NPs or alum as hydropic degeneration in epithelial cells of the renal tubule, necrosis of some epithelial cells in tubules, and obstruction of some of the renal tubules. As depicted in figure 2B, these improvements were more considerable in the kidney of mice at week 8 post-immunization.

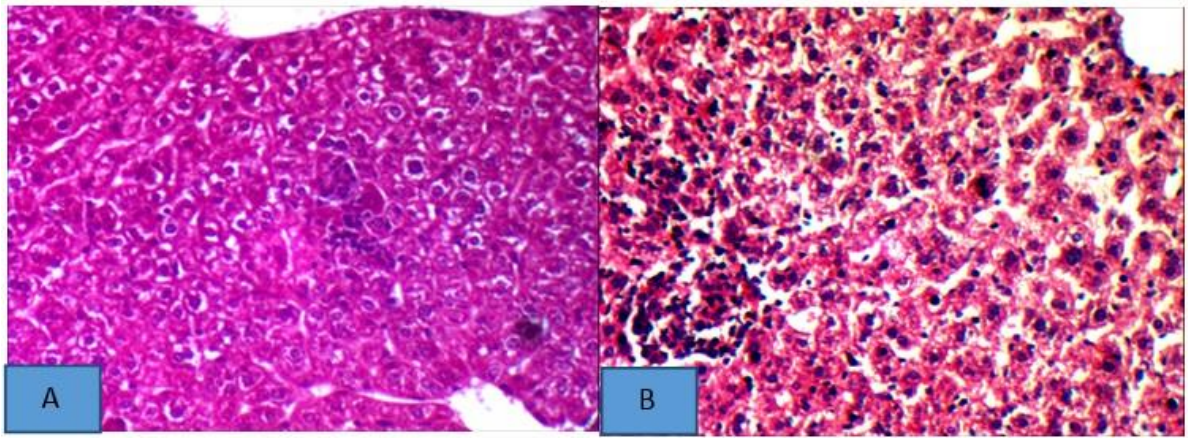

Figure 1. Light micrographs of the liver sections: (A, B**)** adult mouse immunized using aluminum hydroxide adjuvant one month post-vaccination illustrating a hepatocellular necrosis lesion as replaced by mononuclear cells (H & E, × 400).

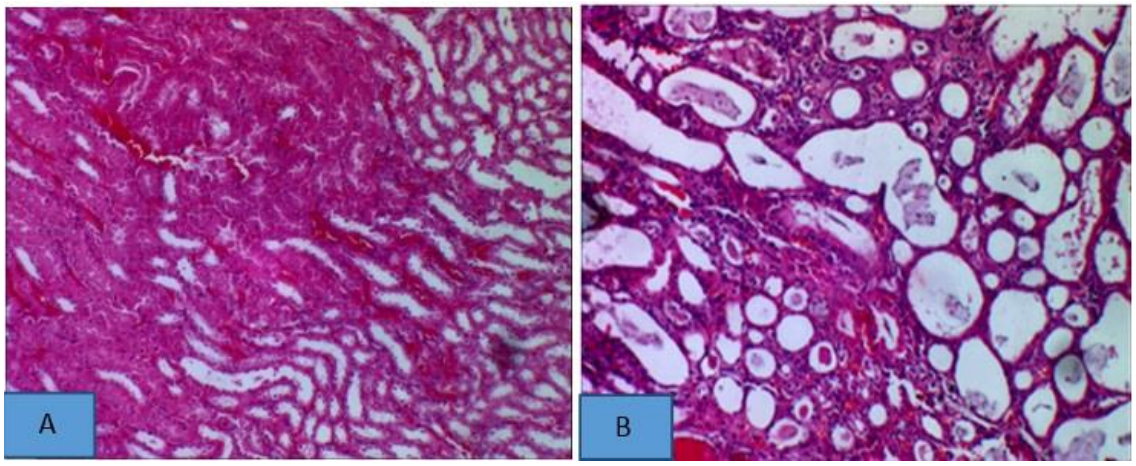

Figure 2. Light micrographs of the kidney sections: A) aluminum hydroxide nanoparticles group indicates renal damage represented by renal tubule epithelial degeneration with distinct infiltration of mononuclear cells. B) aluminum group reveals cytoplasmic vacuolation and distorted renal corpuscles as a result of hypertrophy degeneration of the tubules. Moreover, this figure depicts some vascular glomeruli apparently enlarged filling the Bowman's capsule.

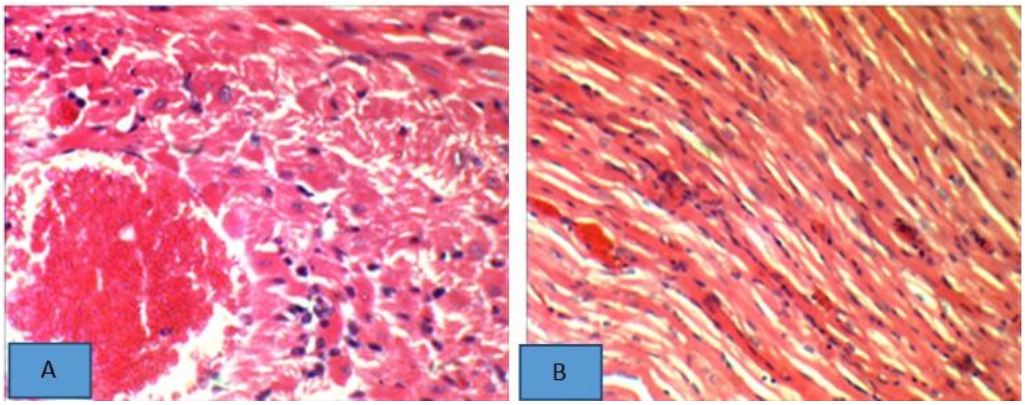

Figure 3. Light micrographs of the heart sections: In aluminum and aluminum hydroxide nanoparticles group (A, B), H & E stain shows myocyte damage, represented by hypertrophy, degeneration, and infiltration of mononuclear cells (×400).

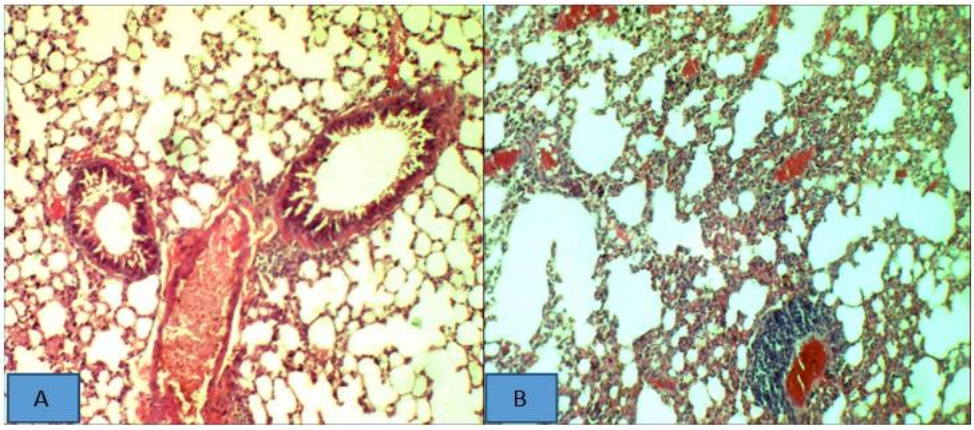

Figure 4. Light micrographs of the heart sections: Aluminum and aluminum hydroxide nanoparticles group (A, B) revealed the congested blood vessels with the thickening of the alveolar wall.

The histopathological changes in the heart in alum (Figure 3A, 3B) and alum hydroxide NPs group revealed myocyte damages, including hypertrophy, degeneration of myocyte (vacuolization), and infiltration of mononuclear cells, while in the lung tissue, there was the congested blood vessel with the thickening of the alveolar wall (Figure 4A, 4B).

3.2. Concentration of DNA

The concentration (ng/µl) of the total DNA in 1 µl of the sample was measured using Fluorometer from Promega (Figure 5; Table 1). It can be concluded that the concentration of DNA in the aluminum adjuvant groups was lower than that in the alum hydroxide NPs groups. This might be due to several reasons, such as decreased protein synthesis, impaired nucleic acid metabolism, cell and tissue damage, inhibition of enzymes responsible for the replication of DNA, and the loss of cell structure.

Figure 5. Quantification of the extracted DNA using Fluorometer from Promega

Table 1. Total DNA content (ng/µl) in the tissue

Groups	ng/µl
Bulk Al $(OH)_3$	0.53
Al $(OH)_3$ nanoparticles	12
Control	2.08

4. Discussion

Although vaccines have a profound impact on global health, several questions still persist about their potential roles in terms of autoimmune or other adverse reactions (9). To address these questions, some components of the vaccine, such as immunogens and adjuvants, require critical assessment for healthy subjects, as well as their safety. It should be mentioned that in the current research, alum and alum hydroxide NPs were prepared and employed as adjuvants.

In the case of both alum and NPs, the histological changes in the liver were severe, such as mononuclear infiltration, hepatocellular necrosis, hydropic degeneration, and activation of Küpffer cells. This issue reveals that NPs activate the sinusoidal cell phagocytic activity by increasing the number of Küpffer cells in order to help remove the accumulated NPs (10).

It should be noted that hepatocyte swelling may occur because of membrane function disruptions, which may be confirmed by the subsequent leakage of lysosomal hydrolytic enzymes (11). Meanwhile, the hydropic degeneration is due to the unbalance in sodium/potassium ions, as well as the fluid homeostasis, leading to an increase in intracellular water (cell swelling) (12). On the other hand, the histological changes that occurred in the kidneys were pronounced in cases of alum adjuvant, including the infiltration of mononuclear cells, vacuolation of the renal tubule epithelium, and congestion of peritubular capillaries, whereas the changes in the case of adjuvant NPs were restricted to the appearance of mononuclear cell aggregation. More importantly, the monocyte infiltration to the kidney is known to correlate with proteinuria and kidney damage (13).

In terms of the alum adjuvant, the histological changes observed in both groups were well noticed and were more apparent at week 8 post-immunization. Moreover, it has been reported that as the NPs can pass through biological blood-brain membranes, they can affect most cells' physiology, such as the brain and tests (14). They may induce increased cell arrests in S, S/(G2/M), as well as the increased apoptotic populations, by interfering with the structure and function of the mitochondria (14).

The obtained results explored the toxic effects on the various tissues (liver, kidneys lung, heart). This could be interpreted due to the small size of NPs that give higher permeability to cell membranes, which consequently affects the cells and cause physiological changes in the investigated tissue (15).

The existence of hemorrhage refers to the endothelium damage induced by a direct toxic effect of the adjuvant used. Furthermore, the infiltration of monocyte revealed that these foreign molecules were recognized using the immune system as a toxic material (15). It is worth mentioning that the current research confirmed the toxicity of Al $(OH)_3$ mediated by systemic inflammatory responses and DNA damage. The toxic effect of NPs was not limited to a certain site. The same factor, the small particle size, allows them to move from the site of application/effect to the other parts of the body. They can pass through the small intestine to the blood, from which, they can move to the brain, lung, heart, kidney, spleen, and liver (16). According to the results of the pharmacokinetic studies, orally administered engineered NPs are mainly toxic to the lungs, liver, and kidneys (17).

All NPs have a significant effect on the liver, lung, heart, and kidney functions, compared to many previous studies. Further confirmation of Al $(OH)_3$-induced damage in organs mentioned earlier was carried out by histopathological sections, indicating the congestive dilution of central veins, as well as focal hepatocellular necrosis. In addition, it showed edema and hepatocyte degeneration. Regarding the renal

tissues, the histopathological section revealed renal epithelial tubule degeneration, vacuolation, distortion of the renal corpuscles, reduction of capillaries in the glomerulus with capsular space, and cellular infiltration in both proximal and distal tubules with pyknotic nuclei.

In this regard, several mechanisms have been proposed to participate in NPs-induced cytotoxic effects. The toxic effect may be mediated by excessive production of reactive oxygen species (ROS), such as hydrogen peroxide, radical hydroxyl compounds, nitric oxide or superoxide anion, oxidative stress, and lipid peroxidation. In addition, genotoxicity and inflammation could be further mechanisms. In this concept, it has been reported that alum hydroxide NPs can interact with both proteins and enzymes. Additionally, they may improve ROS production, resulting in the disturbed antioxidant defense mechanism. It should be mentioned that the initiation of an inflammatory response and disturbed mitochondrial homeostasis are additional factors (18, 19).

According to a study conducted by Klotz and Sies (20), the absorption of metal oxide NPs may interfere with metal ions that cause the loss of these ions by direct interaction with NADPH oxidases (an enzyme that is produced from the plasma membrane or mitochondria resulting in the disrupted electron transport chain as well as producing a superoxide free radical).

The obtained results indicated that the NPs have been shown to respond to oxidizing stress resulting in the activation of pro-inflammatory mediators, such as interleukins (IL-1, IL-6, and IL-8), and macrophage inflammatory protein at both mRNA and protein levels. In this regard, Hou, Xie (21) proposed that when NPs reach the bloodstream, they find a complex web of immune cells and plasma proteins. The identification by the immune cells of NPs as foreign particles would result in the generation of ROS and altered cytokine levels.

This study revealed Al $(OH)_3$-induced apoptosis in hepatic and renal tissues, necrosis in myocytes, and severe damage pulmonary system. According to Patil, Adireddy (22), as well as Bustani and Baiee (23), cell death is attributed to the ROS induced by nanotoxicity. Furthermore, Al $(OH)_3$-induced mitochondria induced oxidative stress and cytotoxicity in the human mesenchymal stem cells (24, 25).

These data showed an oxidative stress status induced by NPs in the liver, lung, heart, and kidneys accompanied by extensive DNA fragmentation. It should be noted that DNA damage is a key event in cellular toxicity. As such, it can be considered an indicator for various NPs-induced increased risk of cancer. Nevertheless, the degradation of DNA is a late event following cell breakage in necrosis. Proteases and endonucleases digest chromatin into a smear pattern but not into a ladder pattern. In other words, the histone is destroyed by protease enzymes, in which the entire length of DNA is exposed to nucleases. Therefore, there is a significant reduction in DNA content of alum adjuvant groups, compared to alum hydroxide NPs groups. Meanwhile, the assessment of cell death by measuring apoptosis and/or necrosis markers specifically implies the capability of NPs to induce cell damage. According to Jennifer and Maciej, Al $(OH)_3$ can induce hepatocyte genotoxicity and cytotoxicity. Therefore, it is essential to understand how NPs can affect the cellular genome in order to interpret the extent of toxicity extent of nano-products. The toxicity of NPs to cells or organs can be described at the molecular level by studying the gene expression that is involved in different cellular processes. Since the mitochondria are an important organelle in cell metabolic processes, ATP production, ROS generation, apoptosis, and any disturbance in mitochondrial homeostasis may be key factors in various pathological and toxicological processes. For further findings, the infarction of cardiac cells and pneumonia (due to the thickening of the alveolar wall) can be addressed.

Furthermore, all of these anomalies seem to have an adverse impact on the alum-treated mice. Therefore, such results are consistent with the findings of the previous studies (26, 27). The mechanism(s) by NPs

can induce hepatic and kidney cytotoxicity, which is of other scientific importance.

Authors' Contribution

Study concept and design: B. H. F.
Acquisition of data: H. A. H. A.
Analysis and interpretation of data: A. M. B. A.
Drafting of the manuscript: B. H. F.
Critical revision of the manuscript for important intellectual content: H. A. H. A.
Statistical analysis: A. M. B. A.
Administrative, technical, and material support: B. H. F.

Ethics

The study protocol was approved by the Institutional Animal Care and Use Committee, Faculty of Veterinary Medicine, University of Kufa, Kufa, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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