Pathological effect of Aluminium hydroxide compared with polymer-based nanoparticles on neonatal mice brain

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Abstract
The present study carried to investigate the toxic pathology effect of both bulk Al(OH)3 and Al(OH)3 nanoparticles materials. Also, to examined cerebral cortex-kb p65 expression level by immunohistochemistry test. The crystalline and grain size of nanoparticles and it is morphology are tested by XRD, AFM, SEM, and EDEX respectively. This study included 40 neonatal mice of both sexes were randomly divided into 7 groups. This group's immunized subcutaneously, two times, the first dose was at third postnatal, and the second dose was at 17 days postnatal. These groups classified as follows:-the 1st group of mice injected by normal saline which serves as control negative, while the 2nd group immunized by oval albumin which serves as control positive. The 3rd group immunized by bulk-Al(OH)3, the 4th group immunized by Al(OH). The Brain tissue samples were collected from each at 4 weeks and 8 weeks post first immunization for histological examination. The Histopathological results of the 1st group, 2nd group do not show clear histopathological changes. Whereas, histopathological changes of the 3rd group was severe at 4 weeks post first immunization, while at 8 weeks post first immunization pathological changes were more severe. However, histopathology changes of the 4th group were less severe (moderate) in 4, 8 weeks post first immunization compared with the 3rd group. The immunohistochemical test results of the 3rd group show a highly significant increase (P < 0.05) (70.2%, 95.6 %) in 4, 8 weeks post first immunization. Whereas, the results of the immunohistochemical test of the 1st and 2nd group, show a significant difference (p<0.05) at 4, 8 weeks post first immunization. While the 4th group shows a significant difference (61%) (P<0.05) in 8 weeks post first immunization compared with Bulk-Al (OH)3. These results demonstrated the toxic effects of bulk Al (OH)3 on the neonatal brain. This study suggested that the chitosan alone and presence with Al(OH)3 in nanomaterial or bulk form safer and less toxic as an adjuvant. In conclusion, it could be concluded that repeated exposure of neonatal mice to Bulk-Al(OH)3 induced histological alterations in various areas of the brain. In addition, results further showed that the expression of NF-kB p65 is significantly increased in the brain of Bulk-Al(OH)3 mice when compared with the control groups. Results of mice immunized with Al(OH)3, NPS severity of toxicity was less than bulk- Al(OH)3. Accordingly, it was concluded that Al adversely affected the brain by histological, immunohistochemical. These alterations are dangerous because of neonatal exposure to aluminum in life.

Key word: Al (OH)3,NPs,BulkAl(OH)3,, Neonatal mice , NF-kb p65,histopathology


Introduction
Nanotechnology is a fast-growing science has applications in diverse fields, combining engineering with biology, chemistry, medicine, and physics and erases the traditional boundaries between them and also it gives the ability to observe and control individual atoms and molecules, and deals with structures in the size range from approximately 1 to 100 nanometers, known as the nanoscale. One nanometer (nm) is a billionth of a meter, or 10-9 meters (1). Nanotechnology, also a rapidly developing science since the last decade of the 20th century and large breakthroughs have been made in the design and manipulation of materials at the nanoscale to impact their performance in biomedical applications.

Several types of substances, including proteins, chemical drugs vaccines can be delivered by nanomaterial-based delivery systems to meet the standards of high bioavailability, sustained and controlled release profiles, targeting, imaging and so forth (2). Aluminum compounds such as alum, aluminum hydroxide, and aluminum phosphate salts were used in vaccines for a long time as an assistant in 1926) (3, 4). Aluminum

compounds are neurotoxic for animals and humans, the toxicity of Al is directly linked to its bioavailability. In biological as long as vaccine systems, this element showed accumulate in many mammalian tissues such as brain, liver, bone, and kidney. Several authors studied that there were neuropathological, neurobehavioral, neurophysical and neurochemical changes after Aluminum exposure.

Aluminium hydroxide nanoparticles used as an immunologic adjuvant because it can produce a high degree of protein adsorption performance also possesses many advantages such as, large surface area, negligible cytotoxicity, and highly effective drug loading to compare with bulk aluminium hydroxide. Also, nano-vaccines are homogenized, which is an important property to stimulate the immune response. So that, aluminium hydroxide in Nano-scale form, can be considered as an advanced technique to promote adjuvant influences. The previous researcher studied that nano-scale form of aluminium adjuvants possess a stronger ability to stimulate a more cellular and humoral immune response in comparison to traditional alum.

Materials and methods

Synthesis of Al(OH)₃-NPs by a chemical precipitation method

The Synthesis of Al(OH)₃-NPs was done by modification of the method. At the first, prepared sodium hydroxide solution by dissolved (39.6) g form NaOH in (110) ml of deionized water in a beaker. Raw material was dissolved in sodium hydroxide solution on a magnetic stirrer at (115)oC for (30) min to get the sodium aluminum solution. The solution was then cooled down to room temperature and diluted with (220) ml of deionized water. (1.43) g of (PEG) was added to the diluted sodium aluminum solution. The sodium aluminum solution was aged for (5) h to fabricate the Al(OH)₃ precipitate. Then, the Al(OH)₃ precipitate was washed by deionized water and ethanol more than one time by using a centrifuge and dried at 100°C for (8) h. Then grinding Al(OH)₃ powder by a milling machine to obtain Al(OH)₃ nanopowder.

Characterization techniques

The Al(OH)₃ nanoparticle solution thus obtained was characterized and used for XRD, SEM, and EDX analyses. The phase formation of synthesized Al(OH)₃ nanoparticles was studied with the help of XRD. The diffraction data of thoroughly dried thin films of nanoparticles on glass slides were recorded on the D8 Advanced Bruker X-ray diffract meter with Cu Kα (1.54 Å). Scanning Electron Microscope (SEM) analysis was done using a Hitachi S - 4500 SEM machine. Thin films of the sample were prepared on a carbon-coated copper grid by just dropping a very small amount of the sample on the grid, and then the film on the SEM grid was allowed to dry.

Adsorption of protein antigens on aluminum hydroxide and chitosan.

The adsorption of proteins oval albumin (OVA) on aluminum hydroxide particles was carried out by mixing the particles in suspension with the protein in solution was added into a tube (10 mg OVA) followed by the addition of particle in suspension at a weight ratio of 1:5 to 1:1 (OVA vs. particles). After 20 minutes of gentle stirring, the protein-particle mixtures were stored at 4°C or freeze-dried if needed before further use. The dose of the OVA was 10 μg per mouse per injection; 20 (or 50) μg per mouse per injection for the particles. Sterile PBS or OVA (10 μg) dissolved in PBS were used as controls, oval albumin diluted with bulk-aluminum hydroxide (Himedia, India) or nanoparticles or chitosan to obtain a final concentration of Adjuvant of 0.1μg/ml.

Dosage and administration

The aluminum injection schedule was intended to mimic the 2010 US pediatric vaccination schedule to maintain consistency with our previous work. The approximate amount of aluminum in all those pediatric vaccines containing aluminum adjuvant at different ages in preschool children. The current study focused on the effects of aluminum on one key characterizing feature of ASD, namely anomalous social interaction. The dosage of Al(OH)₃ adjuvant injected in mice was approximately equivalent (μg/kg) to Al exposure through pediatric vaccines in children.

Experimental animal

Ten pregnant female mice were included in this study obtained from the National Center of Researches and Drugs Monitor in Baghdad. Following impregnation, the females were monitored for the parturition date, 40 neonatal mice (BALB/C) of male genders which were taken as postnatal day (PND) weighted between 5-7 gm divided into 4 groups (ten per group) were subcutaneously immunized with different formulation at a dosage of 0.1 μg. Following impregnation, the females were monitored for the parturition date, 40 neonatal mice (BALB/C) of both genders which were taken as postnatal day (PND) weighted between 5-7 gm divided into 4 groups (ten per group) were subcutaneously immunized with different formulation at a
Result and Discussion

X-ray Diffraction (XRD) Studies

XRD pattern of Al(OH)₃ nanoparticles (Fig. 1), shows a pure hexagonal with a polycrystalline structure. The full width half maximum and grain size of Al(OH)₃ nanoparticles (NPs) from XRD listed in (Table 4.1). The Al(OH)₃ NPs ranging from (31.7 to 72.2) nm with average was 42.3854 nm and XRD spectrum shows split peaks were observed (28.3, 37.5, 47.02, 48, 453.02, 57.5, 63.5).
AFM investigation of Al(OH)$_3$ nanoparticles
According to the Atomic Force Microscope (AFM) test result showed two and three-dimensional topography. The average grain size of nanoparticles was 53.46 nm with a spherical shape with homogenous distribution through the glass substrate (Fig. 2 a, b).

Figure 1: XRD of Al(OH)$_3$ nanoparticles.

Figure 2: Surface structure of Al(OH)$_3$ Nanoparticles. a: Two dimensional, b: Three dimensional Surface structure characterized by AFM technique.

Scanning Electron Microscope
According to Scanning Electron Microscope test result showed the average particles size nanoparticles 37.27 nm with spherical shape with homogenous distribution through the glass substrate (Fig 3).
Figure 3: SEM photomicrograph of Al(OH)₃NPs with 10µm magnification, the inset is SEM image with 5mm magnification.

Histopathology of brain of neonatal mice 4weeks, 8weeks post first immunization.

The microscopic examination of group one mice did not show any histological changes in the cerebral cortex, hippocampus, and cerebellum 8weeks post first immunization. Eight weeks post first immunization brain mice of group two showed absence of pyramidal cell due to degeneration lead to form spaces in cerebral parenchyma (Fig. 4 a), while cerebellum showed absence of Purkinje cells in cerebellum parenchyma (Fig. 4 b). Eight weeks post first immunization brain mice of group four showed neural degeneration with pyknosis and gliosis in the cerebral cortex (Fig. 5 a). The cerebellum showed mild disruption in the Purkinje cells in cerebellum parenchyma (Fig. 5 b). Results of Statistical Analysis of Histopathological change in brain mice immunized with Bulk-Al(OH)₃ showed a significant difference (P<0.05) in 8 weeks post first immunization when compared with other groups. Severity lesion of brain mice immunized with Al(OH)₃NPs showed significantly difference (P<0.05) when compared with normal saline showed significant difference (P<0.05) in 8 weeks post first immunization. Mann-Whitney Test used to study the significant difference between groups.

Figure 4: Photomicrograph of brain of neonatal mice immunized with Bulk-Al(OH)₃(H and E 200x). a: cerebral cortex section showed absence of pyramidal cell due to degeneration lead to form of spaces in cerebral parenchyma was observed 8weeks post first immunization, b: cerebellum section showed Purkinje cells are hardly found in the Purkinje cell layer was observed 8weeks post first immunization.
Immunohistochemical results.
Mild positive NF-κB p65 expression with percentage 7.2% was recognized in brain mice of group one in 8 weeks post first immunizations (Fig. 6). Eight weeks post first immunization brain mice of group three showed strong NF-κB p65 immunostaining with mean percentage 95.6% (Fig. 7). Brain mice of group five in 8 weeks post first immunizations showed moderate NF-κB p65 immunostaining with mean percentage 61% (Fig. 8). Results of Statistical Analysis of immunohistochemical expression of NF-κB p65 in brain mice of group three showed significant (P<0.05) difference in NF-κB p65 expression when compared with other groups in 8 weeks post first immunization. Brain mice of group four showed significantly (P<0.05) difference when compared with group one at 8 weeks post first immunization.

Figure 5: A Photomicrograph of the brain of mice immunized with Al(OH)₃ NPs (H&E 200x). a: cerebral cortex section showed neural degeneration with pyknosis and gliosis in 8 weeks post first immunization. b: cerebellum showed mild disruption in the Purkinje cell in cerebellum parenchyma was observed 8 weeks post first immunization.

Figure 6: Photomicrograph of Cerebral cortex of mice immunized with normal saline. The cerebral cortex showed a significant decrease in NF-κB p65 expression at 8 weeks post first immunization (400×).

Figure 7: Photomicrograph of the cerebral cortex of mice immunized with bulk-Al(OH)₃. Cerebral cortex showed a very significant increase of NF-κB p65 expression at 8 weeks post first immunization (400 magnification).
Figure 8: Photomicrograph of Cerebral cortex of mice immunized with Al(OH)3NPs. Cerebral cortex showed a significant increase of NF-κB p65 expression at 8 weeks post first immunization (400 magnification).

Discussion

The current study was conducted to investigate the toxicity effect of Al(OH)3 in bulk and nano form. Al(OH)3 NPs were prepared by the chemical precipitation method resulted in the size of a nanoparticle of 42.3854 nm was investigated by using XRD. However, the pattern of prepared Al(OH)3 nanoparticles is indexed as a pure monoclinic phase (spacegroup: P21/n) (JCPDS No. 33-0018), the narrow sharp peaks indicate that Al(OH)3 nanoparticles are well crystallized and broaden peak refers to the synthesis of nanostructure (12,13). However, Atomic force microscope analysis found the average grain size of nanoparticles was 53.46, with a spherical shape with homogenous distribution through the glass substrate (16,17).

Scanning electron microscopic analysis shows nanoparticles have a spherical shape with homogenous distribution through the glass substrate, and the white big shape of a particle refers to aggregation due to Van Der Wals band and this means the solution needs more sonication, this result agrees with AFM result (11).

Aluminum hydroxide is the more widely used and is found in vaccines against tetanus, hepatitis A, hepatitis B, Haemophilus influenza B, pneumococcal and meningococcal infections, and anthrax (19). There are many investigations about Aluminum toxicity neurotoxicity in some experimental animals (19, 20, 21). Although aluminum hydroxide may deleteriously impact various organ systems, some of its worst impacts may be on the nervous system (13, 14, 22). Bulk- Al(OH)3 immunized mice cause toxicity in 8 weeks post first immunization. was showed severe pericellular edema, diffuse gliosis, necrosis of neuron, neuronal degeneration and vacuolation, presence of neurofibrillary tangles in cerebral cortex. Previous studies showed that other aluminum compounds were toxic in the brain such aluminum chloride (23,24). Cerebellum showed a significant reduction in the number of Purkinje cells Disorganization of the Purkinje cell layer with the absence of the Purkinje cells was previously reported with aluminum exposure (27). The few observed Purkinje cells in this study showed a darkly stained cytoplasm and dark (pyknotic) nuclei. Pyknosis was described as irreversible condensation of chromatin in the nucleus of the cell undergoing programmed cell death or apoptosis (28). Ultra structurally some Purkinje cells were shrunken with an electron-dense cytoplasm and ill distinct nuclei whereas others showed rarified nuclei with faint outlines like a nuclear ghost, some. Changes observed in this study could be explained by the effect of reactive oxygen species (ROS) generated by Aluminum exposure. This exposure resulted in high mitochondrial membrane potential and elevated levels of oxidized proteins and lipids (29). Also, AlEefy et al. (22) reported exposure to aluminum hydroxide resulted in marked degenerative effects on the rat’s testis.

The histopathology result of mice immunized with Al(OH)3 nanoparticles showed the severity of toxicity was less toxic than Bulk- Al(OH)3, and was able to reverse this pathology considerably; this was shown by the complete absence of eosinophilic amyloid plaques with the present an occasional neurofibrillary tangle, moderate neuronal degeneration and vacuolation in cerebral cortex, hippocampus and cerebellum, presence of edema and vacuolated neuron cytoplasm in 8 weeks post first immunization.

The previous researcher showed other nanoparticles cause histopathological changes in tissues of the liver, kidney, brain and injected muscle (30). The primary mechanism of nanoparticle toxicity involves the production of reactive oxygen species (ROS) and free radicals. (34). The nanosized particles oxidize the neural membrane that lost its lipoprotein integrity and partially cause damage to the blood-brain barrier, and in turn, facilitates the accumulation of Al to the brain tissues. The accumulated aluminum attaches to the mitochondria and/or the nuclei causing the damage of the cell and degradation of neurons (32). Researchers
have conducted many in vivo and in vitro studies to explore the interactions between the nanomaterials and biological macromolecules, cells, organs, and tissues, and the majority of these studies have found that the effects of the biological toxicities of the nanomaterials may be induced by the mechanisms of oxidative stress and inflammatory reactions (32, 33).

Role of chitosan in the decrease of NF-κB p65 activation and decrease toxicity Transcription factor NF-κB is known to have a prominent role in the inflammatory response (35, 36). Several studies have indicated that the expression of cytokines, chemokines, and adhesion molecules, such as TNF-α, MCP-1, RANTES, fractalkine, iNOS, and ICAM-1, is governed by NF-κB (36, 37, 38). Inhibition of NF-κB reduces from inflammation. The current study’s objective was to evaluate the expression of these key molecules in the brain of mice. The NF-κB pathway is implicated in brain inflammation. Among its family members, p65 is the most frequently studied. It is part of the p65 (RelA):p50 dimer which mediates the classical pathway.

In the present study, p65 was expressed in the cytoplasm of the brain cells in every case. Bulk Al(OH)₃ immunized mice in 8 weeks post first immunization the toxicity was severe in brain expression of cytoplasmic NF kb p65 in the brain was significantly high 31.00% (p<0.05). This could be explained by the fact that Aluminum, being a non-physiological metal, accumulates in the body and is dispersed in different regions of the cell. The major sites of localization are the mitochondria, lysosomes, and nucleus in the cell. It has been shown that Aluminum accumulates in neurons following cell depolarization (39). This step requires a release of 1kB form NF-κB through phosphorylation by 1kB kinase (40). This is in accordance with Chunyaxiner et al., 2014, who found elevated NF-κB p65 level in aluminum-induced brain toxicity. As well as on Queet at (39) speculated that Al causes significant in NF-κB p65 expression.

NF-kB has also been reported to be involved in the survival of cerebellar granule neurons subjected to different potassium concentrations (42). In global ischemia and traumatic spinal cord injury, however, NF-kB promotes neuronal death (43). Thus, it appears that whether NF-kB acts as a promoter or inhibitor of neuronal loss depends on the cell type and the nature of the toxic stimuli. Aluminum accumulation directly associated with ap regulation of the proinflammatory transcription factor NF-Kb and the inflammatory post-transcriptional epigenetic regulator microRNA-146a in the same tissues. Activation of this aluminum – ROS-NF-Kb-miRNA-146a pro-inflammatory genetic circuit and in amyloid and cytokine stressed human brain cells in primary culture it marks the onset of neurodegeneration (40). Al(OH)₃ NPs immunized mice showed 8 weeks post first immunization showed a significant difference in NF-κB p65 expression detected in 20.50%. The severity of toxicity was less in 8 weeks than bulk Al(OH)₃ which may be attributed to Al(OH)₃ NPs have minimized toxic effect on mice brain (46). Nanoparticles stimulated reactive oxygen species (ROS) production, which may be both related and not related to NPs-induced cellular senescence.

Indeed, the role of ROS in cellular metabolism is much more complex than previously thought (46, 47). Of course, when the level of ROS is high, the impairment of redox homeostasis may lead to oxidative protein and DNA damage and a concomitant apoptotic/necrotic cell death. However, ROS at the moderate levels is considered molecular secondary messengers regulating cellular signaling pathways (48). ROS may modulate redox reactions affecting active sites of enzymes and the activity of transcription factors, such as NF-kB, JUN, and FOS (49).

Reference
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