Evaluation Of The Histopathological, Immunohistochemical, And Oxidative Neurotoxic Effects of Nanoaluminum Present In Human Vaccines In Newborn Albino Rats

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Abstract

Background: The increasing prevalence of autism spectrum disorder and Alzheimer's disease is associated with the amount of nanoaluminum in various national paediatric immunisation regimens. **Objectives:** The study aimed to evaluate the possible neurotoxic effects of nano aluminum used in human vaccines on newborn albino rats including histopathology, immunohistochemistry (IHC) and oxidative stress and to assess the prophylactic effect of nano curcumin in brain toxicity **Methods:** Injections of a “high” and “low” Al adjuvant levels were designed to correlate to either the U.S. or Scandinavian pediatric vaccine schedule in Group 1and 2 respectively vs. control saline-injected rats (Group 5). Groups 3 and 4 have taken the same injection as groups 1 and 2 respectively plus nanocurcumin orally. **Results:** Elevation of MDA levels and decreased SOD levels in groups of rats treated with aluminium nanoparticles with increased apoptosis, as indicated by high levels of monoclonal antibody BAX, decreased levels of anti-apoptotic monoclonal antibody BcL-2, and a high BAX/Bcl2 ratio in rats receiving aluminium adjuvants. Increased inflammation of cells in brain tissue by an increased level of iNOS monoclonal antibodies. We found cut-off values for iNOS and Bax that can discriminate group 1 from group 5 and group 2 from group 5. Those values are 215, 216.5, 200 and 202, respectively, with sensitivity = 100% and specificity = 100%. Nanocurcumin can cause marked improvements in neurotoxicity, apoptosis, monoclonal antibodies, and oxidative stress caused by nano AL adjuvants. **Conclusion:** Nanoaluminum adjuvants can cause several side effects, which nanocurcumin can prevent.

Keywords: nanoaluminum, aluminium adjuvants, vaccination, neurotoxicity, nanocurcumin

Introduction

Aluminium is the most frequent metal and the third most frequent element in the Earth's crust (Al). Al is often chemically linked to other elements; therefore, it is not usually accessible and appears to have unknown biochemical function in plants, animals, or humans. Al, however, has increased significantly in the human environment during the past 150 years due to human activity. Al is a substance that is widely utilized in processed foods, several pharmaceuticals, and water treatment. It is also widely employed in

industrial and material applications. Due to its prevalence, it might be found in our bodies more frequently. **(Tomljenovic et al., 2011).**

One of medicine's greatest achievements, immunizations protect millions of people from getting sick and passing away before their time. Along with the essential antigens, inactivated and attenuated vaccines, such as the Salk polio and tuberculosis vaccines, also contain adjuvants consisting of natural immune stimulants. Exogenous adjuvants must, however, be added to vaccination formulations, such as tetanus, diphtheria, and pertussis vaccines, that contain separated and purified antigen. Aluminum salts have been used for years to achieve this goal in order to encourage an adaptive immune response to co-administered antigens **(Danielsson et al., 2018).**

Aluminum adjuvants are added to several vaccines to elicit a more robust immune response and increase vaccine efficacy. In the United States, Canada, Europe, Australia, and many other parts of the world, infants and young children receive high quantities of aluminum from multiple inoculations. For example, in the U.S. the hepatitis B, DTaP (for diphtheria, tetanus and pertussis), pneumococcal (PCV), Haemophilus influenzae type b (Hib), and hepatitis A vaccines are all administered during early childhood. Each of these vaccines contains aluminum, and booster shots in repeated doses are needed. At age 2 months, infants receive an immediate injection of 1,225 micrograms of aluminum; by age 18 months, they have collected 4,925 micrograms of aluminum **(Miller, 2016, Goldstein, 2017).**

Aluminum oxyhydroxide and aluminium hydroxyphosphate are the most common and current forms of aluminium salts utilised as adjuvants in vaccine formulations. Although both forms of aluminium adjuvants are made up of nanoparticles that aggregate into free, micrometre-sized particles at a pH of 7, their physical and chemical characteristics are very different **(Johnston et al., 2002).**

Recently, there has been a significant correlation between the amount of aluminium (Al) used in various national paediatric immunisation programmes and the increased prevalence of autism spectrum disorder (ASD); this correlation appears to be dose dependent. The so-called Hill causality criteria were satisfied by this ecological data on 8 or 9 **(Shaw et al., 2013).**

It has been suggested that aluminium in immunisations may cause autism. For instance, research revealed a highly significant positive linear association between babies' exposure to aluminium through vaccinations and the prevalence of autism in numerous affluent countries (Pearson r = 0.89-0.94**) (Tomljenovic and Shaw, 2011).** In a different research, Shaw et al. discovered that some children may be more susceptible to the harm that paediatric immunisations with aluminium cause to their central nervous systems due to genetic predispositions **(Shaw et al., 2014).** Moreover, vaccines containing aluminium adjuvants are administered directly into the body, avoiding the digestive system and skin's natural defences. This route of aluminium absorption is more effective than oral absorption, which increases the risk of harmful effects. "Data has now emerged showing that autism may in part originate from early-life immunological insults generated by environmental xenobiotics," the scientists concluded in a summary of their research. The aluminium vaccination adjuvant is one of the most prevalent xenobiotics with immunostimulant and neurotoxic effects to which infants under two years of age are regularly exposed globally **(Miller, 2016).**

Blood-brain and blood-cerebrospinal fluid barriers are breached by aluminium from vaccine adjuvants, leading to detrimental immuno-inflammatory reactions in neural tissues. Yet, despite knowledge that aluminium is hazardous to both humans and animals, clinical investigations on vaccination safety frequently administer injections containing aluminium to a "control" group as a safe "placebo." Aluminum cannot be used as a placebo, and this is unacceptable. Given that several doses of infant immunisations are virtually uniformly given, data plausibly link aluminium adjuvants to rising rates of autism spectrum disorders in those nations. Young mice were given high or low doses of aluminium adjuvants (intended to correspond with American or Scandinavian childhood immunisation regimens) in a different recent animal study **(Shaw et al., 2013).** Notable alterations in the mice were seen, supporting the idea that aluminium adjuvants have a deleterious effect on the central nervous system. These current observations indicate aluminium infused in early postnatal life in some central nervous system abnormalities that may be relevant for a better understanding of the genesis of autism spectrum disorders," the authors wrote in response to their findings **(Miller, 2016).**

Children from countries with the highest ASD prevalence, such as the United States, Canada, and Egypt, were shown to have a substantially higher exposure to aluminium through vaccines than children from nations with a lower ASD prevalence (i.e., Scandinavian countries). Also, children in Scandinavia receive less vaccines overall and later in life than children in North America, in accordance with their own immunisation recommendations (**Shaw et al., 2013).**

Understanding how to regulate particles at the nanoscale, their behaviour, and their attributes are the main goals of the extremely promising field of nanotechnology **(Yousef et al., 2019).**

Numerous researchers from throughout the world have investigated the pharmacological effects of curcumin. It is possible for curcumin to reduce both acute and chronic inflammation. By lowering histamine levels and perhaps enhancing the adrenal glands' synthesis of natural cortisone, it minimizes inflammation **(Baum and Ng, 2004)**. Curcumin also demonstrated anti-inflammatory activity on human vascular cells in in vitro tests. Curcumin's anti-inflammatory effects are caused by its ability to interfere with NF-B, which reduces the inflammatory response of human endothelial cells that have been activated by TNF-. Additionally, curcumin has the ability to inhibit platelet-derived growth factor (PDGF) **(Kim et al., 2007).**

Curcumin has a considerable ability to scavenge superoxide radicals, hydrogen peroxide, and nitric oxide (NO) from activated macrophages, reduce iron complex, and decrease lipid peroxidation, according to a number of studies. Both in vitro and in vivo studies have demonstrated that it can scavenge a variety of reactive oxygen species produced by macrophages, such as superoxide anions, hydrogen peroxide, and nitrite radicals **(Joe et al., 2004).** These processes could be the primary means by which curcumin demonstrates its antioxidant properties.

Potentially, curcumin aids in the prevention of newly developed malignancies brought on by radiation or chemotherapy. Through its impact on a number of molecular processes involved in mutagenesis, oncogene expression, cell cycle regulation, apoptosis, tumor igenesis, and metastasis, curcumin has recently been discovered to possess anti-cancer properties **(LoTempio et al., 2005).** Curcumin's anti-tumor-promoting properties have been examined and shown in numerous studies. Due to the stimulation of apoptosis in human leukemia cells, it was demonstrated in these experiments that curcumin has antitumor-promoting properties **(Kuo et al., 1996).** Studies have revealed that dietary curcumin specifically inhibits cyclooxygenase (cox) - 2 in human colon cancer cells and human breast carcinoma cells **(Goel et al., 2001; Shao et al., 2002).**

There is an antibacterial effect of turmeric. In vitro, curcumin reduces the growth of Helicobacter pylori strains as well as other bacteria like streptococci, staphylococci, lactobacillus, etc. Curcumin inhibits Streptococcus mutans' ability to produce cariogenic substances at doses between 0.5 and 4 mg/ml. It works well as a root canal medication in endodontics and is also effective against Enterococcus faecalis **(Neelakantan et al., 2013).** As it is effective against Aspergillus flavus, Aspergillus parasiticus, Fusarium moniliforme, and Penicillium digitatum **(Moghadamtousi et al., 2014),** it also functions as an antifungal agent. It is anti-protozoan and effective against Plasmodium falciparum, Leishmania, and E. histolytica.

One of the many medicinal properties of turmeric is an antihyperalgesic action. Activating the transient receptor potential vanilloid 1 (TRPV1), which is crucial for nociception, requires the vanilloid moiety of curcumin, according to a study. The transient receptor potential vanilloid 1 (TRPV1), a key player in nociception, is thought to be activated by the vanilloid moiety of curcumin. The findings further highlight how curcumin suppresses TRPV1-mediated pain hypersensitivity by competitively blocking capsaicin's activation of TRPV1 **(Yeon et al., 2010).**

The main compound in turmeric, curcumin, has a variety of positive pharmacological effects that include neuroprotection due to its antioxidant, antidepressant, anti-inflammatory, and anti-cancer properties. However, curcumin's therapeutic application is constrained by factors such as low bioavailability, poor water solubility, low plasma and tissue concentrations, instability in body fluids, and restricted gastrointestinal absorption. More bioavailability and an enhanced therapeutic effect on the brain could be accomplished using nanotechnology and breaking down curcumin to the nanoscale **(Mourtas et al., 2014).** The bioavailability and therapeutic effectiveness of curcumin have been found to enhance when it is broken down to nano size, according to numerous research **(Sookhaklari et al., 2019).** Nanocurcumin was

effective in reducing Parkinson's disease PD symptoms in a fly PD model **(Siddique et al., 2014).** In mice brains, nanocurcumin was discovered to have a greater bioavailability than curcumin and to have a protective impact against oxidative stress **(Nazari et al., 2014).** In comparison to natural curcumin, nanocurcumin has been demonstrated to have a longer mean residential duration in mice brains **(Cheng et al., 2013).**

The goal of our study is to look at the possible toxic effects of nanoaluminum used in human vaccines on the brains of new-born albino rats using histopathology and immunohistochemistry (IHC) and to see if nanocurcumin can prevent brain toxicity.

Materials and Methods

Study design & experimental animals

This an experimental observational case control study. The study included 50 new-born rats divided into five groups, each consisting of 10 animals according to the calculated sample size. New-born albino rat's day 2 post-natal, and both sexes were included. Adult albino rats were excluded. We distributed the injection schedule in albino rats over their first three postnatal weeks since the first three weeks in albino rats roughly equate to the human equivalent of 0–6 years of age as rats were weaned at around 5–6 weeks of age, when they attained sexual maturity (similar to a post-puberty in humans, i.e. 12–15 years). (This is, of course, an approximation based mostly on life duration and certain elements of early postnatal neuro development may differ greatly between humans and rats). We spaced out the schedule of injections in rats during the first three postnatal weeks because the majority of pediatric vaccines are administered to children before the age of 6 following **Shaw & Tomljenovic (2013)** study. Water and food were freely available to all animals.

Sample size:

The sample size analysis was executed using GPower software version 3.0.10. According to the design of the experiment, the suitable statistical test will be one way analysis of variance (ANOVA) to study the effect of one factor with five levels on the studied parameters. A total number of 40 rats (8 rats per each group) will be required to gain a test power of 80%, at $\alpha = 0.05$ and effect size = 0.7. The effect size was based on a previous study by Morsy et al. (2016). We used 10 rats in each group for possibility of mortality during the experiment.

Ethical considerations

The Medical Science Sector, Kasr Alainy ethical committee, and Cairo University Institutional Animal Care and Use Committee (CU-IACUC) all approved the study's design (IRB: CU-III-F-62-20).

Experimental design:

Table 1 shows how much nanoaluminum is in human vaccines and how much is injected into mice according to US and Scandinavian schedules **(Tomljenovic, 2011).**

The duration of administration of aluminium adjuvants and nanocurcumin was 17 days. G1: According to the US schedule "High AL," the injection regimen was shown in ***Table 2.*** G2: According to the Scandinavian schedule "Low Al," the injection regimen was shown in ***Table 2.*** **G3**: Received the same schedule as Group 1, but the injection was combined with oral administration of 100 mg/kg of nanocurcumin. **G4**: Received the same schedule as Group 2, but the injection was combined with oral administration of 100 mg/kg of nanocurcumin. **G5**: The saline control was injected with 15 l of saline at days 2, 4, 9, 11, and 16 postnatally. In the end, 9 animals persisted in all groups except group 2, where only 8 animals persisted, despite the fact that we had originally planned to utilize 10 animals in each testing group because of worry that some might not survive. For each animal the total injection volume was 15 µl of either saline alone or Al hydroxide in saline. The rats were anaesthetized with ketamine hydrochloride (50 mg/kg) by the end of the 17 days after finishing all dosages, and they were killed by cervical dislocation. The injection was subcutaneous in a defined area near the neck (the least painful area in their body) despite the vaccines given intramuscular in human.

Sample Collection

Brain samples were collected for further estimations. Half of the samples were preserved in formalin for histopathological examination and immune histochemistry examinations, while the other half was frozen and stored at -80 °C for the detection of oxidative markers.

Chemicals

AL hydrogel®, as a source of nanoscale aluminum hydroxide, a gel suspension of aluminium hydroxide (AlOH3) was employed. This gel's composition is thought to be similar to that found in commercially available vaccines. It was bought from the Sigma Aldrich Company. Alhydrogel® adjuvant 2%, also referred to as alum, is an aluminum hydroxide wet gel solution. Alum causes a Th2 response by increasing the antigen's attraction to and absorption by antigen-presenting cells (APCs). Furthermore, pattern recognition receptors can activate innate immune pathways (PRRs).

Alhydrogel® particles display a net positive electrical charge at pH 5-7, making them particularly well suited for the adsorption of negatively charged antigens. (For instance, antigens with formulation pH-below isoelectric values)

Nanocurcumin was purchased from Nanotech Co. The purity of the chemicals used is of reagent grade (≥ 95 %). Appearance (Color): Yellowish Brown. Appearance (Form): Powder. Capping Materials: PVA Solubility: Suspension in Water & Colloidal in Ethanol Optical Prop. (Abs.): λmax = 425 nm Size (TEM): 50 ± 5.5 nm Shape (TEM): Spherical Like-shape ***(figure 1).***

Detection of an oxidative stress marker by colorimetry kit

Total Superoxide Dismutase (T-SOD) Activity Assay Kit (Hydroxylamine Method) SOD was measured in brain tissue homogenate by using a T-SOD ELISA kit provided by Elabscience, Catalog No.: E-BC-K019-S (USA). MDA was measured in brain tissue homogenate by using a T-SOD ELISA kit provided by Elabscience, Catalog No. E-BC-K025-S (USA).

Histopathological examination

The brain samples were preserved in 10% formalin, then dehydrated, cleaned, and embedded in blocks of paraffin. Hematoxylin and eosin (H&E) was used to make paraffin sections of 5 mm thickness, which were then inspected microscopically for congestion and edema. *Immunohistochemistry (IHC)*

1. The presence of inflammation is detected by the expression of a monoclonal antibody (isoform nitric oxide synthase—iNOS).
2. Apoptosis detection markets include pro-apoptotic, Bax protein and anti-apoptotic Bcl2 protein, as well as monoclonal antibodies for apoptosis activators.
3. Bax/bcl2 ratio:

The link between the apoptosis promotor Bax and the apoptosis inhibitor Bcl2 was calculated in the current study. The Bax/Bcl2 ratio determines a cell's propensity to go through apoptosis **(Fadl et al., 2020).** Brain tissue immunostaining was done to look at how much the iNOS, Bax, and Bcl2 proteins were expressed. The tissue samples were formalin-fixed and paraffin-embedded for 5 mm before being cut into sections and placed on silane-coated slides. For specimens that were evaluated as standard sections, slides were dewaxed and rehydrated. In an autostainer, immunohistochemical staining was carried out (Dako autostainer link 48). Antigen retrieval was carried out in a hot 10 mm sodium citrate buffer at pH 6.0, gradually increasing from 50°C to 100°C in a microwave oven for 40 min. The following primary antibodies were used:

1. Bax monoclonal antibody (E-AB-22212) (1:100, IHC-P),
2. Elabscience Biotechnology, USA, produced the BCL2 monoclonal antibody (E-AB-22004) (1:50, IHC-P).
3. Rabbit polyclonal anti-iNOS antibody (ARG56509) (1:100, IHC-P). The antibodies were purchased from Arigo.

Biolaboratory: Mouse spleen tissue that had been paraffin-embedded served as a positive control. The identical procedure was followed while processing negative controls, with the exception of using the primary antibody. iNOS, Bax, and Bcl2 each had the area percentage of immunohistochemical expression examined quantitatively. Six non-overlapping fields were used to generate the data for each slide in each group. **(El Asar et al. 2019)**.

Statistical Analysis

The statistical package for the social sciences (SPSS) version 28 was used to code and enter the data (IBM Corp., Armonk, NY, USA). For quantitative variables, mean and standard deviation were used to summarize the data. For categorical variables, frequencies (number of cases) and relative frequencies (percentages) were used. Analysis of variance (ANOVA) with multiple comparisons and a post hoc test was used to compare the groups. Chi square (2) test was used to compare categorical data. When the anticipated frequency was less than 5, an exact test was utilized in its place. The Pearson correlation coefficient was used to calculate correlations between quantitative variables. To find the appropriate cut-off value of important parameters for group differentiation, an area under the curve analysis was used to generate a ROC curve. Statistics were considered significant for P-values under 0.05.

Results

The macroscopic images display different degrees of congestion and hemorrhage in comparison to the control group: groups G1, G2, and G3 had the highest degree of congestion and hemorrhage, while group 4 had the lowest degree ***(Figure 2).***

Comparing the congestion and edema (inflammation) microscopically between the studied groups, there was a statistically significant (p value 0.001) difference between them. Groups 3 and 4 had mild to moderate inflammation compared to control group 5, whereas group 1 displayed severe inflammation. However, group 2 displayed greater inflammation than groups (3 and 4), but less than group 1 ***(table 3).***

There was a statistical difference between the mean monoclonal antibodies (iNOS, Bcl2, and Bax), which was very significant when comparing these markers between the examined groups (p value 0.001) ***(table 4).***

Compared to the control group 5, group 1 had significantly higher iNOS expression (P = 0.001), while groups 3 and 4 had significantly lower iNOS expression. However, compared to groups 3 and 4, group 2 had significantly higher iNOS expression ***(Figures 3 and 4).***

Group 1 had significantly higher Bax expression than the control group 5 (P 0.001), groups 3 and 4 showed much lower Bax expression, and group 2 had significantly higher Bax expression than groups 3 and 4 ***(Figures 5 and 6).***

Group 4 considerably increased Bcl2 expression (P 0.05) when compared to the control group 5, but group 1 dramatically decreased expression. Bcl2 expression was higher in group 3 than in group 2 in comparison ***(Figures 7&8).***

Group 1 was where the Bax/Bcl2 ratio was found to be at its highest value. The Bax/Bcl2 ratio in group 4 was the lowest, and in groups 3 and 2, it was lower than in the control 5 group, while greater in group 2 than in group 3 ***(figure 9).***

There was a statistical difference in the means of the oxidative stress markers when Eliza compared the studied groups. In comparison to the control group 5, group 1 showed a significantly high MDA level (p value 0.001), whereas in group 4, expression was significantly reduced. Regarding SOD level, group 5 showed a significantly high level (p value 0.001), whereas in group 1, the expression was significantly reduced ***(table 5).***

There was a statistical difference between the mean neurotoxicity markers, which was very significant when comparing these markers between the examined groups (p value 0.001) ***(table 6)***.

Cut-off values between groups 1 and 5:

The cut-off values of the monoclonal antibodies between group 1 and the control group are calculated ***(table 7).*** We found cutoff values for iNOS and Bax. Those values are 215 and 216.5, respectively, that can discriminate group 1 from group 5 with sensitivity = 100% and specificity = 100%. Values above the cut-off are assigned to group 1. On the other hand, Bcl2 cannot be used for discrimination between groups 1 and 5.

Cut-off values between groups 2 and 5:

Cut-off values of the monoclonal antibodies between group 2 and control group 5 were calculated ***(table 8).*** We found cutoff values for iNOS and Bax. Those values are 200 and 202, respectively, that can discriminate group 2 from group 5 with sensitivity = 100% and specificity = 100%. Group 2 includes values above the cutoff. On the other hand, Bcl2 cannot be used for discrimination between group 2 and group 5.

Pairwise comparisons between the groups under study

Some of the changes between the groups were very statistically significant (p values 0.001) using post hoc pairwise p-value comparisons in all markers between each pair of groups, while others were statistically significant (p values 0.05). However there was no statistically significant difference between some of the groups (p-value 0.05) (***Table 9).***

Table1: The calculated total amount of nano aluminium in human vaccines and the equivalent amount injected to mice.

	Birth	2m	4m	6m	15m	2years	6years
• Hepatitis • Diphtheria-pertussis-tetanus • Haemophiles influenza type b • Pneumococcal • Hepatitis A	250	250 375 112.5 125	 375 112.5 125	250 375 112.5 125	 375 112.5 125 250	 250	 375
Total Al (μg) in children	250	862.5	612.5	862.5	862.5	250	375
Total Al (μg/kg bw) in children	73.5	172.5	107.5	113.5	78.4	19.8	19.3
Total Al (μg/kg bw) injected into neonatal rats G1("high Al" group)		170	150	110	80	20	20
Total Al (μg/kg bw) injected into neonatal Rats G2("low Al" group)			90	80	50		50

Table2: Total Al (μg/kg bw) injected into neonatal rats and the corresponding day of injection in groups 1&2.

Group 1	
μg/kg body weight	Post-natal days of rat's life
170	3
150	5
110	6
80	10
20	12
20	17
Group 2	
90	4
80	6
50	10
20	17

Table 3: Congestion and edema within the studied groups using chi square test.

		Group 1		Group 2		Group 3		Group 4		Group 5		P value
		Count	%	Count	%	Count	%	Count	%	Count	%	
Congestion / edema	0	0	0.0%	0	0.0%	0	0.0%	0	0.0%	4	66.7%	< 0.001
	1	0	0.0%	0	0.0%	4	44.4%	9	100.0%	2	33.3%	
	2	3	33.3%	5	62.5%	3	33.3%	0	0.0%	0	0.0%	
	3	6	66.7%	3	37.5%	2	22.2%	0	0.0%	0	0.0%	

0 negative congestion and edema.
1 mild congestion and edema.
2 moderate congestion and edema.
3 sever congestion and edema.

Table 4: monoclonal antibodies within the studied groups (mean ± SD) using One Way ANOVA test

Standard Deviation	G1		G2		G3		G4		G5	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
iNOS	305	18.4	278	16.87	181.67	28.17	154.78	17.06	85.17	33.98
Bcl2	41.78	7.71	55.88	11.84	66.11	16.66	70.67	13.51	43.83	24.14
Bax	318.56	28.98	259.25	17.61	242.89	24.45	201.22	9.97	147.5	25.03

Table 5: Eliza measurements within the studied groups (mean ± SD) using ONE way ANOVA.

	Group 1		Group 2		Group 3		Group 4		Group 5	
	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation
MDA	157.59	14.77	146.13	18.08	57.1	6.79	37.91	6.99	20.27	2.1

(umol/g)										
SOD (U/mg Protein)	0.55	0.04	0.49	0.02	0.68	0.03	0.64	0.04	0.82	0.08

Table 6: Neurotoxicity markers within the studied groups (mean ± SD) using One Way ANOVA test:

	Group 1	Group 2	Group 3	Group 4	Group 5	P value
Inos/P	**305±18.4**	**278±16.87**	**181.67±28.17 *#**	**154.78±17.06 *#**	**85.17±33.98 *#$@**	**<0.001**
bcl2/P	**41.78±7.71**	**55.88±11.84**	**66.11±16.66 ***	**70.67±13.51 ***	**43.83±24.14 @**	**0.001**
Bax/P	**318.56±28.98**	**259.25±17.61 ***	**242.89±24.45 ***	**201.22±9.97 *#$**	**147.5±25.03 *#$@**	**<0.001**
bax/bcl ratio	**7.86±1.58**	**4.89±1.38 ***	**4.01±1.58 ***	**2.98±0.83 ***	**4.1±1.79 ***	**<0.001**
MDA (umol/g)	**157.59±14.77**	**146.13±18.08**	**57.1±6.79 *#**	**37.91±6.99 *#$**	**20.27±2.1 *#$**	**<0.001**
SOD (U/mg Protein)	**0.55±0.04**	**0.49±0.02**	**0.68±0.03 *#**	**0.64±0.04 *#**	**0.82±0.08 *#$@**	**<0.001**

Values are presented as mean ±SD
*: statistically significant compared to corresponding value in group 1 (P<0.05).
#: statistically significant compared to corresponding value in group 2 (P<0.05).
$: statistically significant compared to corresponding value in group 3 (P<0.05).
@: statistically significant compared to corresponding value in group 4 (P<0.05).

Table 7: Calculation of cut-off value for discrimination between high nano aluminium group and control group using Critical Threshold method.

	Area Under the Curve	P value	95% Confidence Interval				
			Lower Bound	Upper Bound	Cut off	Sensitivity %	Specificity %
iNOS/p	**1.000**	**< 0.001**	**1.000**	**1.000**	**215**	**100**	**100**
Bcl2/p	**0.583**	**0.656**	**0.217**	**0.950**	**----**	**----**	**----**
Bax/p	**1.000**	**< 0.001**	**1.000**	**1.000**	**216.5**	**100**	**100**

Table 8: Calculation of cut-off value for discrimination between low nano aluminum group and control group using Critical Threshold method.

	Area Under the Curve	P value	95% Confidence Interval				
			Lower Bound	Upper Bound	Cut off	Sensitivity %	Specificity %
inos/p	**1.000**	**< 0.001**	**1.000**	**1.000**	**200**	**100**	**100**
bcl2/p	**0.656**	**0.412**	**0.283**	**1.030**	**----**	**----**	**----**
bax/p	**1.000**	**< 0.001**	**1.000**	**1.000**	**202**	**100**	**100**
P53 (Fold change)	**1.000**	**< 0.001**	**1.000**	**1.000**	**2.2**	**100**	**100**
UCP2 (Fold change)	**1.000**	**< 0.001**	**1.000**	**1.000**	**3.4**	**100**	**100**

Table 9: Post hoc Pairwise Comparison between studied groups concerning histopathological examination of brain sections.

		Group 1	Group 2	Group 3	Group 4
inos/p	**Group 1**		0.212	<0.001	<0.001
	Group 2	0.212		<0.001	<0.001
	Group 3	<0.001	<0.001		0.183
	Group 4	<0.001	<0.001	0.183	
	Group 5	<0.001	<0.001	<0.001	<0.001
bcl2/p	**Group 1**		0.602	0.014	0.002
	Group 2	0.602		1.000	0.492
	Group 3	0.014	1.000		1.000
	Group 4	0.002	0.492	1.000	
	Group 5	1.000	1.000	0.076	0.016
bax/p	**Group 1**		<0.001	<0.001	<0.001
	Group 2	<0.001		1.000	<0.001
	Group 3	<0.001	1.000		0.003
	Group 4	<0.001	<0.001	0.003	
	Group 5	<0.001	<0.001	<0.001	<0.001
bax/bcl ratio	**Group 1**		0.001	<0.001	<0.001
	Group 2	0.001		1.000	0.098
	Group 3	<0.001	1.000		1.000
	Group 4	<0.001	0.098	1.000	
	Group 5	<0.001	1.000	1.000	1.000
MDA (umol/g)	**Group 1**		0.487	<0.001	<0.001
	Group 2	0.487		<0.001	<0.001
	Group 3	<0.001	<0.001		0.012
	Group 4	<0.001	<0.001	0.012	
	Group 5	<0.001	<0.001	<0.001	0.064
SOD (U/mg Protein)	**Group 1**		0.218	<0.001	<0.001
	Group 2	0.218		<0.001	<0.001
	Group 3	<0.001	<0.001		1.000
	Group 4	<0.001	<0.001	1.000	
	Group 5	<0.001	<0.001	<0.001	<0.001
	Group 5	<0.001	<0.001	0.243	1.000

p-value <0.05 is statistically significant.

p-value <0.001 is statistically highly significant.

p-value >0.05 is statistically insignificant.

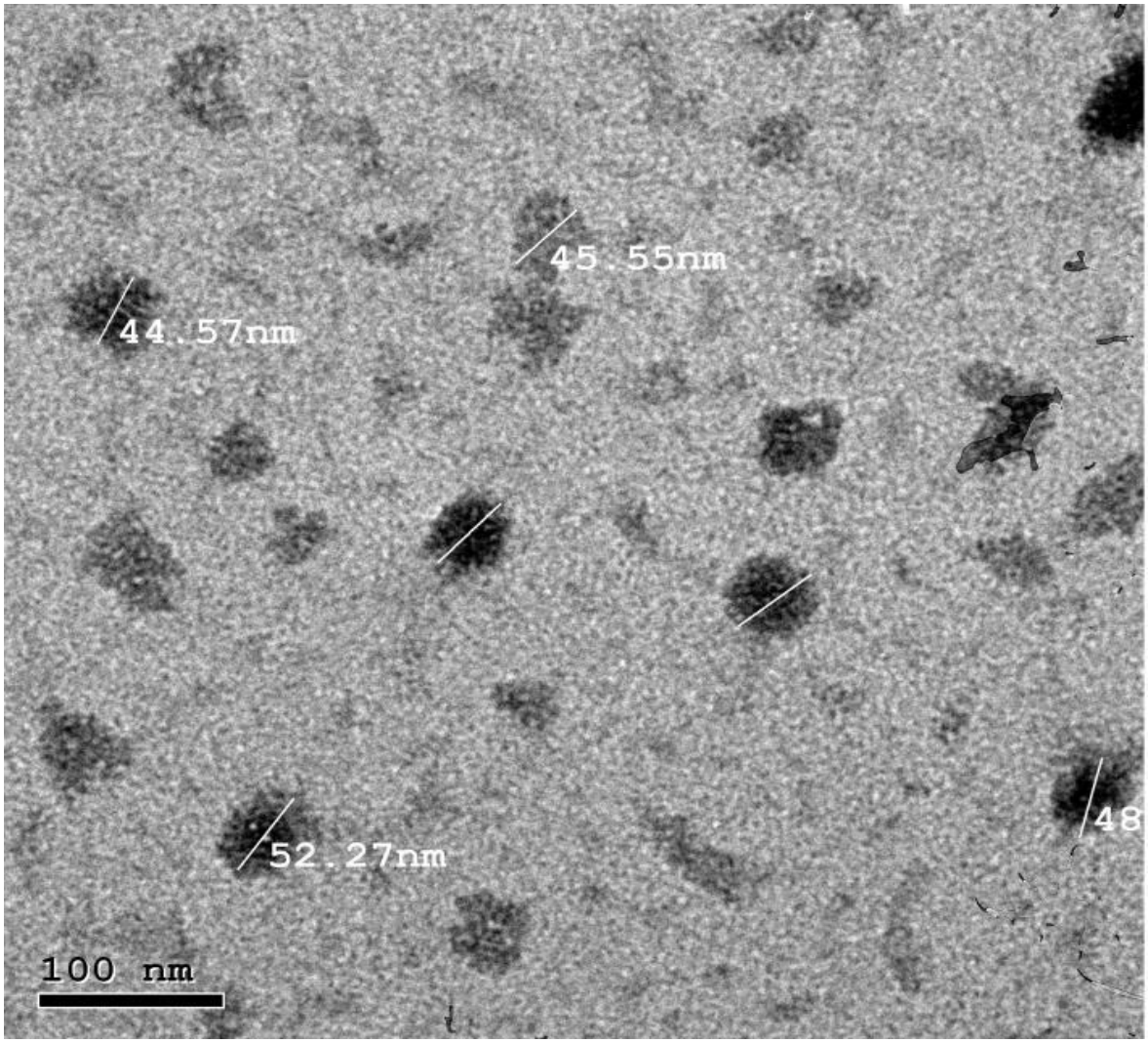

***Figure (1)*:**TEM micrograph for as-prepared PVA capped Curcumin NPs

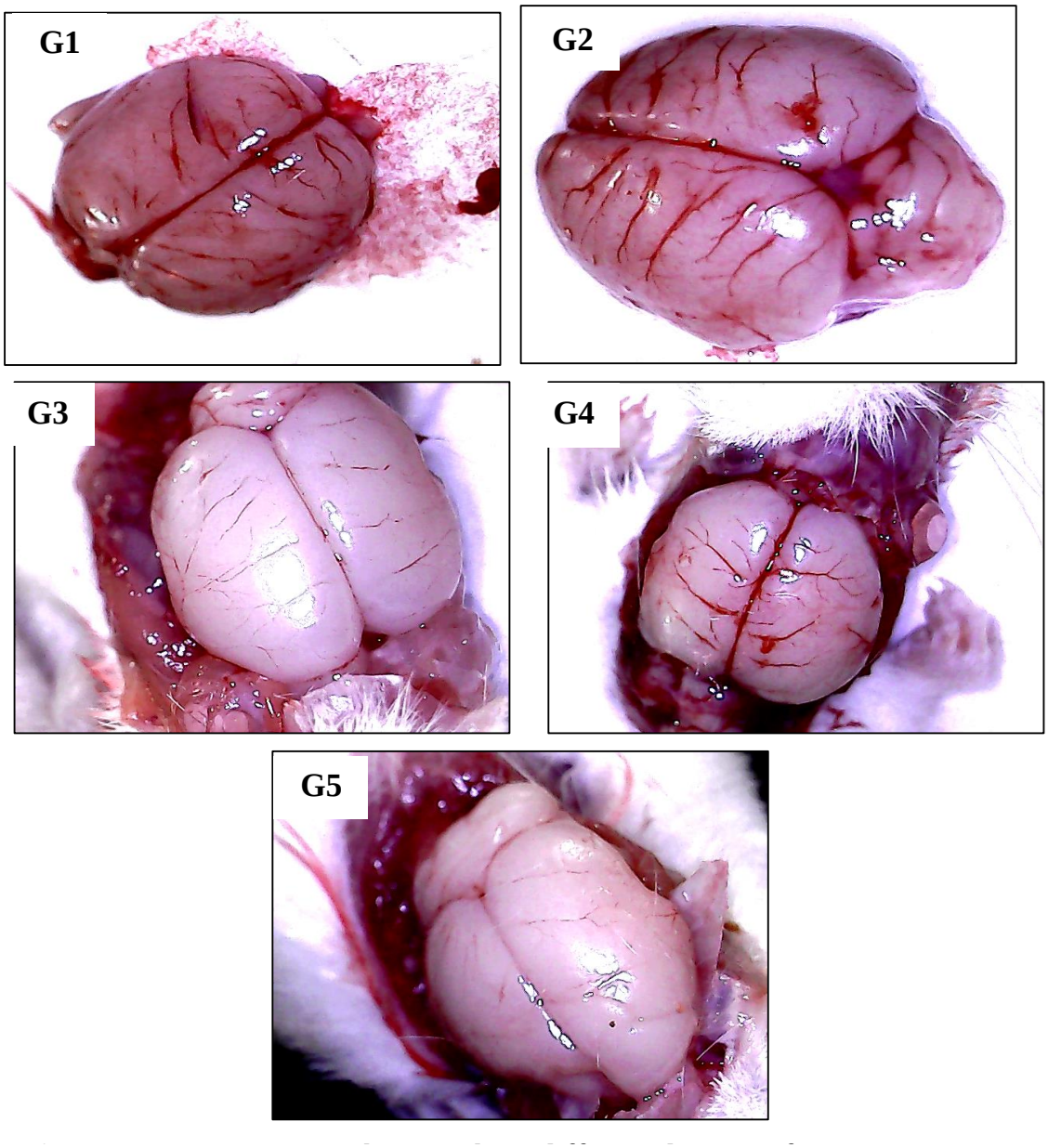

Figure 2: Macroscopic changes show different degrees of congestion and haemorrhage.

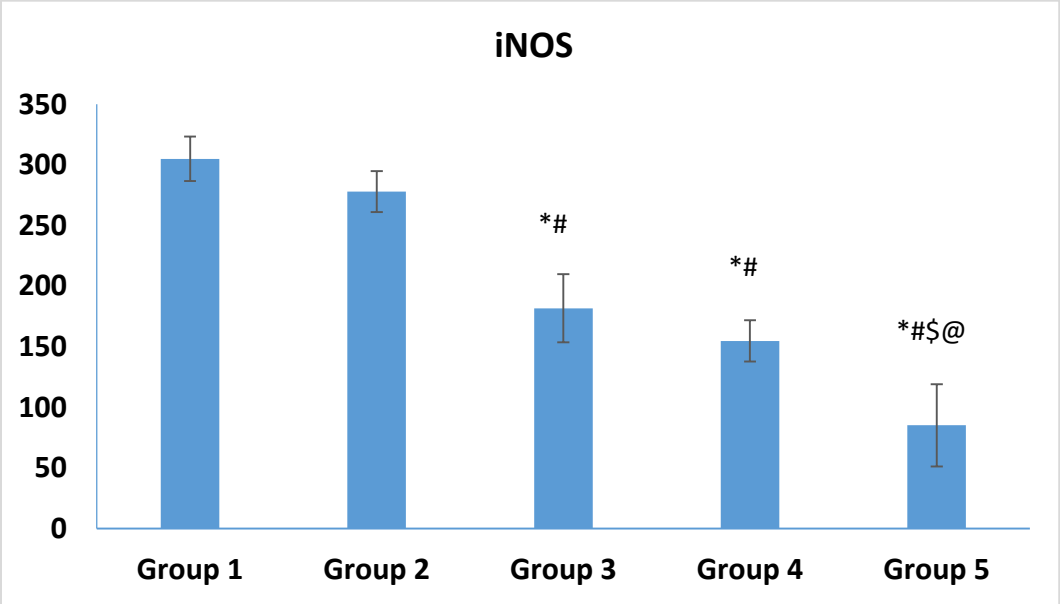

Figure 3: Area percentage of iNOS immunoreactivity in different groups.

*: statistically significant compared to corresponding value in group 1.

#: statistically significant compared to corresponding value in group 2.

$: statistically significant compared to corresponding value in group 3.

@: statistically significant compared to corresponding value in group 4.

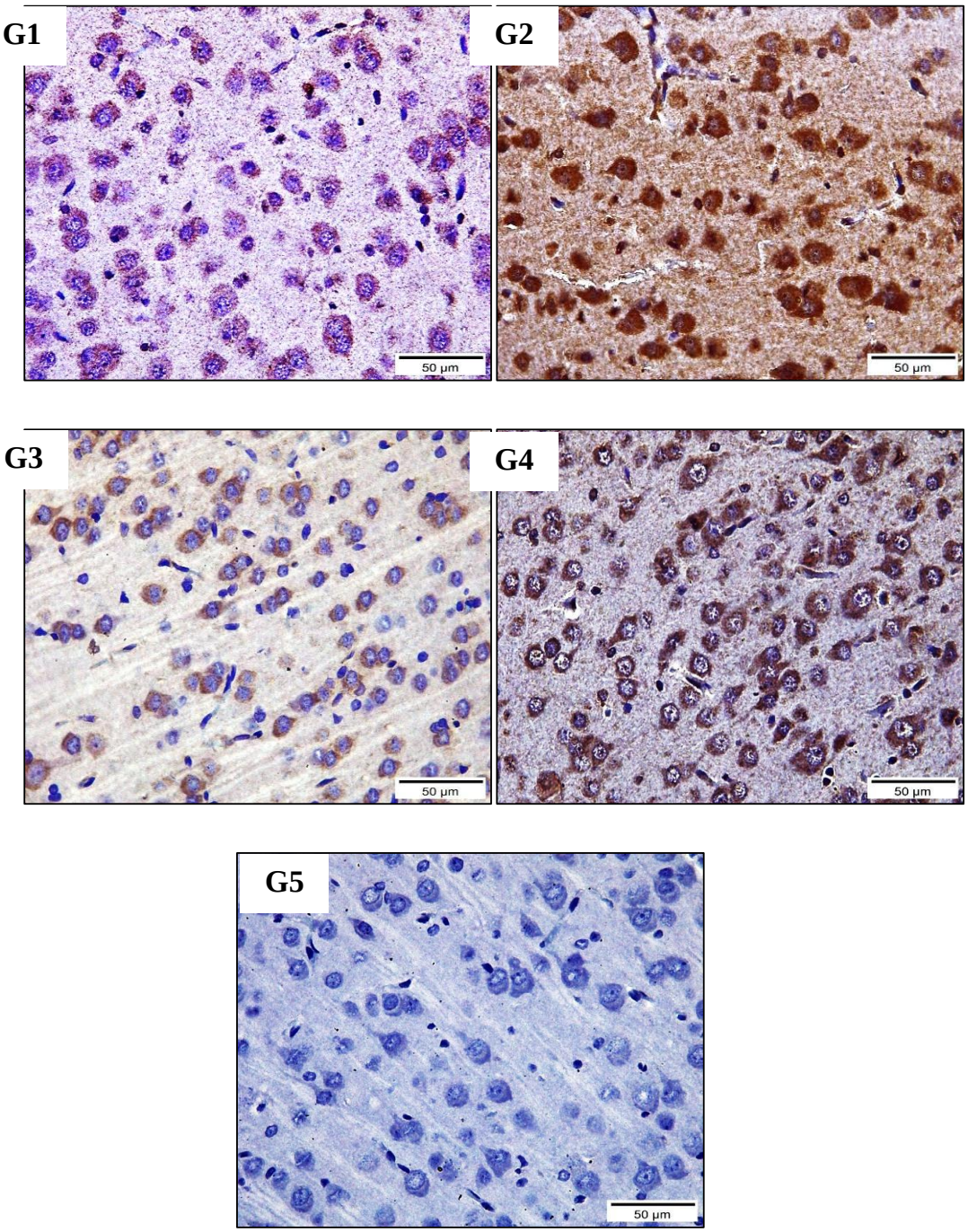

Figure4: *Immunohistochemistry* expression of iNOS in brain tissue (mag. 400) (scale bar 50 µm).

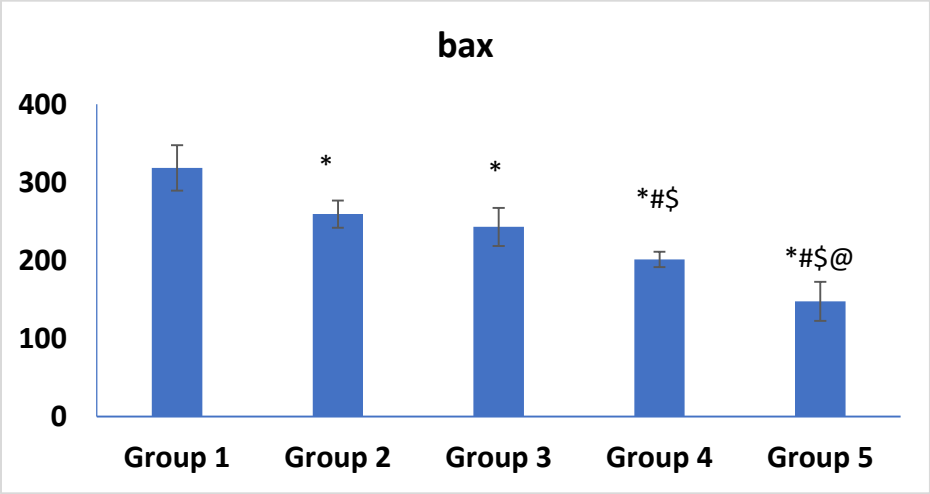

Figure 5: *Area percentage of Bax immunoreactivity in different groups.*
* Statistically significant compared to corresponding value in group 1
# Statistically significant compared to corresponding value in group 2
$ statistically significant compared to corresponding value in group 3
@ Statistically significant compared to corresponding value in group 4

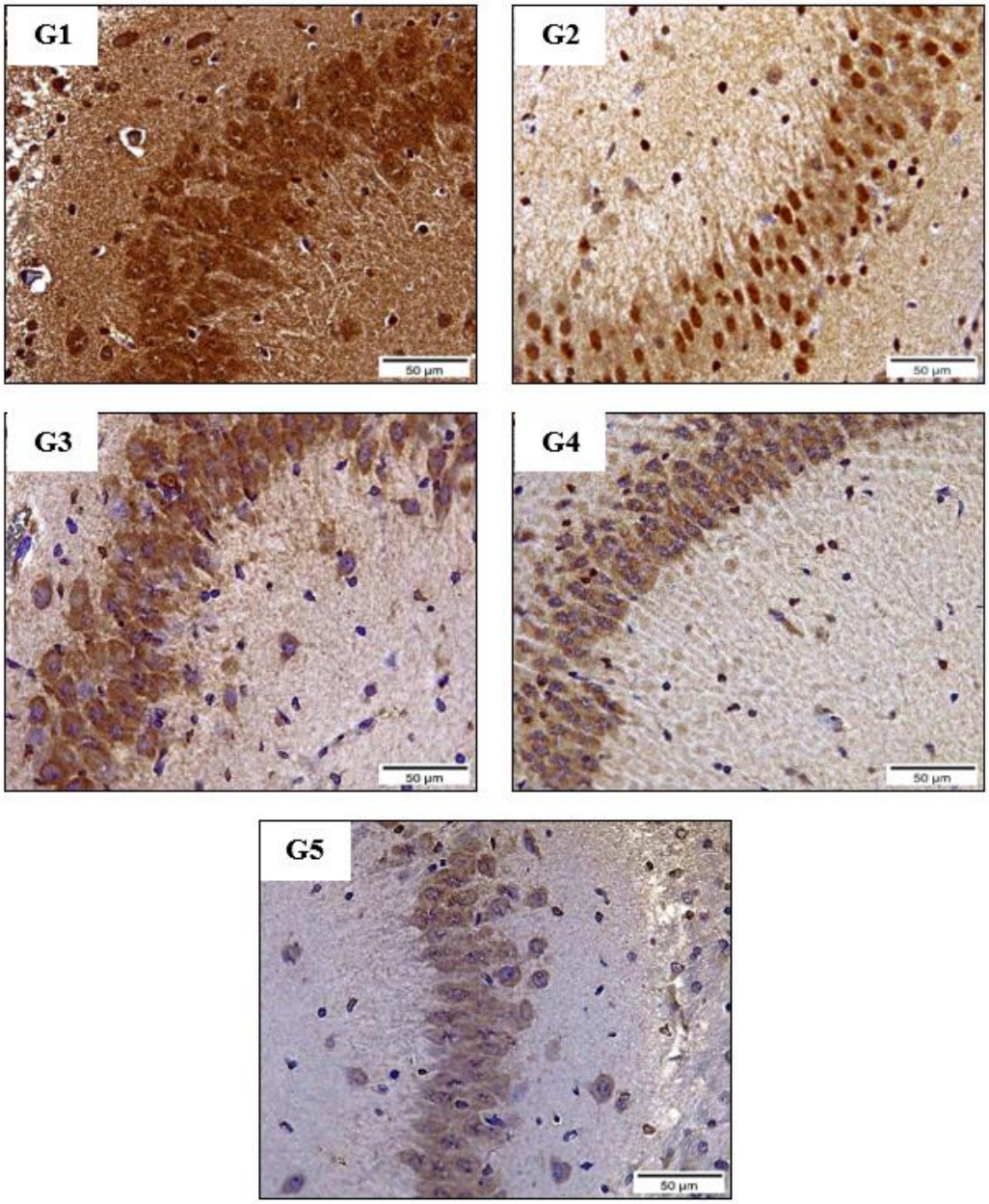

Figure 6: *Immunohistochemistry* expression of BAX in brain tissue (mag. 200) (scale bar 50 µm).

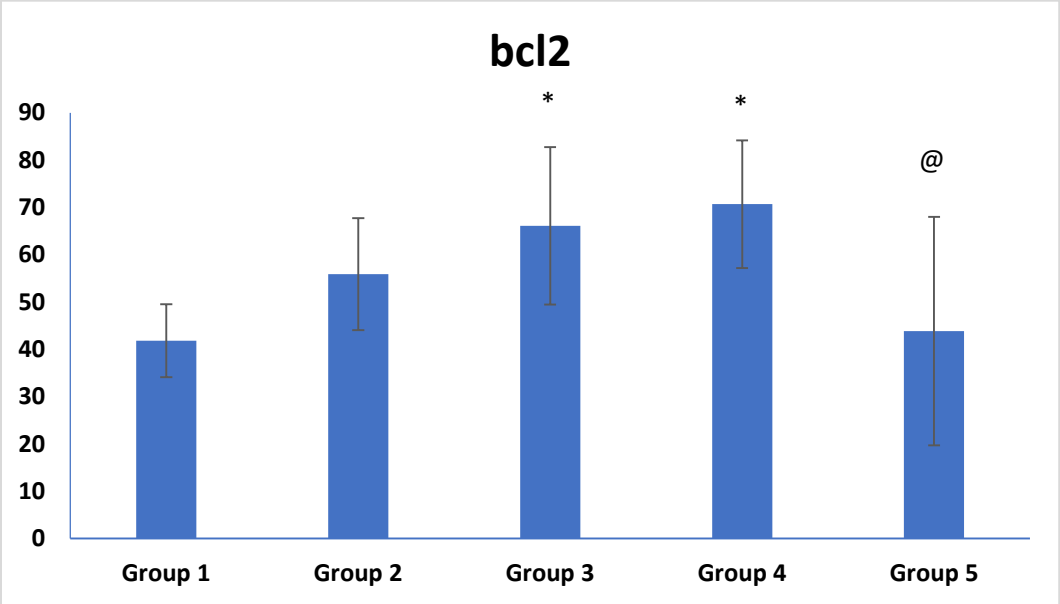

Figure7: *Area percentage of Bcl2 immunoreactivity in different groups.*

* Statistically significant compared to corresponding value in group 3.

@ Statistically significant compared to corresponding value in group 4.

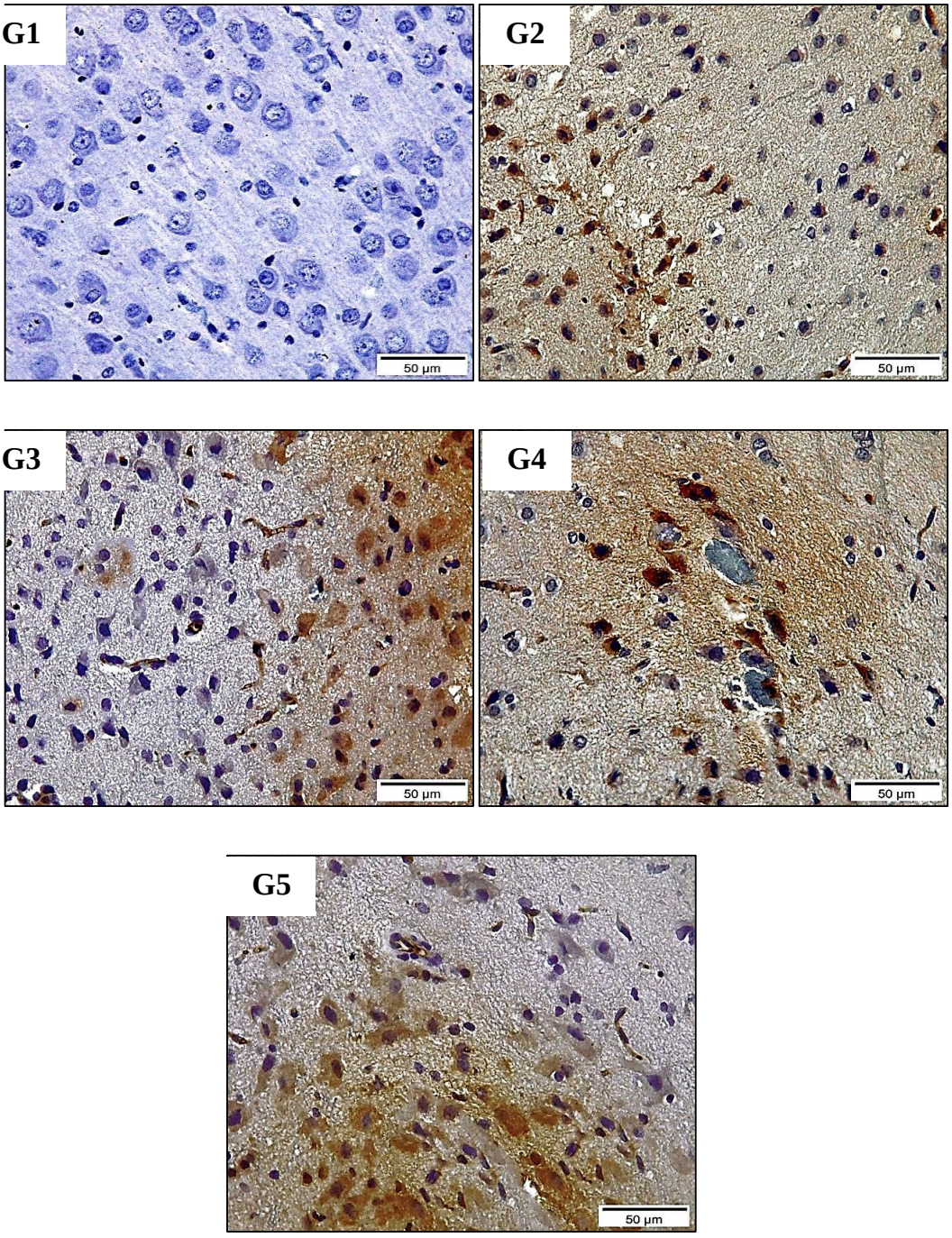

***Figure 8*:** Immunohistochemistry expression of Bcl2 in brain tissue (mag. 400) (scale bar 50 µm).

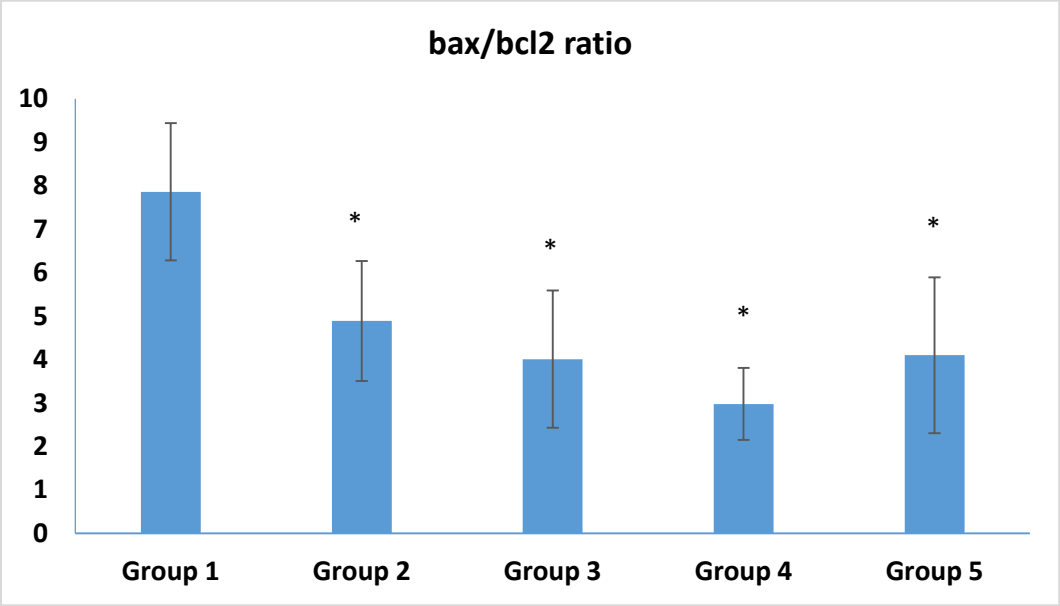

Figure 9: *Bax/ Bcl2 ratio in the different groups*
**: statistically significant compared to corresponding value in group 1.*

Discussion

The current experimental study aimed to determine the neurotoxic effects of nano-aluminium used in human vaccines on the brains of new-born albino rats, explain the mechanism of neurotoxicity, and assess the effects of nano-aluminium exposure on the macroscopic picture, histopathology, immunohistochemistry, including pro-inflammatory, pro apoptotic, and anti-apoptotic monoclonal antibodies, and cell stress by oxidative stress enzymes. We also looked at whether nanocurcumin could operate as a preventative measure against the neurotoxicity brought on by the nanoaluminum present in human vaccines or not.

In a similar study, **Shaw & Tomljenovic (2013)** sought to develop an animal model to investigate potential behavioral traits and central nervous system abnormalities using a similar animal setup but mice instead of albino rats. Aluminum administration during the early postnatal period is linked to a variety of changes in the central nervous system (CNS), some of which could be significant in understanding the factors that contribute to the development of autism spectrum disorder. The animals' pediatric immunization schedules led to large weight gains for both male and female mice in the "high Al" group (ASD).

In our experiment, we discovered that rats of both sexes given injections in accordance with the "high Al" schedule had the maximum degree of congestion and bleeding, whereas rats given injections in accordance with the "low A1" schedule had the lowest degree. The macroscopic images show different degrees of congestion and hemorrhage in comparison to the control group. On the other hand, groups that received the nanocurcumin experienced less hemorrhage and necrosis than those that did not.

This is in line with the findings of **Abou-Zeid et al., 2021** who administered rats with or without oral sesamol (100 mg/kg) for 28 days after gavageing AlNPs (100 mg/kg) to the rats. They found that AlNPs resulted in haemorrhages, edoema, neuronal necrosis, and/or apoptosis in the medulla oblongata.

Mrad et al. (2017) examined the effects of four intravenous injections of Al2O3-NPs (20 mg/kg body weight) on the brains of Wistar male rats. The frontal brain and cerebellum of rats were studied to determine if there was a connection between aluminium oxide nanoparticles and cognitive impairments and oxidative stress. Astrogliosis, vascular obstruction, neuronal degeneration, the presence of oedema and necrosis, degenerative neurofibrillary tangles, and vacuolated cytoplasm were the most obvious histological alterations seen in FC tissue.

Regarding the monoclonal antibodies, in our study, the "high Al" schedule showed significantly higher levels of iNOS, an inflammatory marker, and Bax, a pro-apoptotic marker (P 0.001), while the "low A1" schedule showed increased expression in comparison to the control group but less than the first group. In

contrast, iNOS and Bax expression were significantly reduced in the groups taking nanocurcumin. The "low A1" schedule that took nanocurcumin had significantly higher Bcl2 expression (an anti-apoptotic marker) than the control group (P 0.05), whereas the "high Al" schedule had significantly lower expression. The susceptibility of a cell to apoptosis is increased in high nanoaluminum toxicity more than in low level toxicity, and nanocurcumin reduces this susceptibility of cell breakdown. The "high Al" schedule reported the highest Bax/Bcl2 ratio value. The lowest Bax/Bcl2 ratio was seen in the "low A1" regimen that used nanocurcumin.

Aluminium oxide nanoparticle neurotoxicity and its molecular connection to p53-related pathways, Bax, and Bcl expression were investigated by **Liu et al. in 2020**. They looked at how rats' vitally functioning sub-brain regions were distributed and damaged after receiving intranasal instillations of nano-Al2O3. Additionally, nano-Al2O3 triggered apoptosis, disrupted the cell cycle, and reduced mitochondrial activity. Additionally, nano-Al2O3 boosted the expression of p53, p21, Bax, and Rb while decreasing the expression of cyclin D1, bcl-2, Mdm2, and phospho-Rb.

Using their findings, **Yousef an et al., 2019** administered daily oral dosages of Al2O3NPs (70 mg/kg BW), ZnONPs (100 mg/kg BW), or a combination of the two to male rats for 75 days. ln order to determine whether zinc oxide nanoparticles (ZnONPs) and aluminium oxide nanoparticles (Al2O3NPs) individually or collectively posed a risk to the testicles, sperm, DNA fragmentation, cytokines, oxidative stress, sex hormones, and histopathology of the testes in male rats.

In our results When Eliza compared SOD and MDA (oxidative stressors) between the studied groups, there was a statistical difference between their means that was highly significant (p value 0.001). Regarding MDA, a marker for oxidative stress, it was found at significantly higher levels in the "high Al" schedule than in the "low A1" schedule compared to the control group, but it decreased considerably in the groups receiving nanocurcumin. On the other hand, the SOD, which measures the cell's ability to fight free radicals with antioxidants, was much higher in the groups that got both nanocurcumin and nanoaluminum than in the groups that only got nanoaluminum. This shows that the curcumin protects against the nanoaluminum's oxidative stress.

MDA buildup with age targets mitochondrial enzymes, causing mitochondrial dysfunction and further ageing and neurodegenerative effects. MDA decreased the potential of the mitochondrial membrane and inhibited the complex I- and complex II-linked respiration of the mitochondria in a dose-dependent manner. MDA considerably increased mitochondrial ROS levels. A large dose of MDA marginally lowered mitochondrial superoxide dismutase in terms of the antioxidant defence system (SOD). The findings imply that MDA-induced neuronal mitochondrial damage may have a significant impact on both brain ageing and neurodegenerative diseases **(Long et al., 2009).**

Manke et al. (2013) demonstrated that the ROS-mediated damage brought on by engineered NP is actually caused by cellular responses such as mitochondrial respiration, NP-cell interaction, and immune cell activation as opposed to oxidative stress, which is brought on by acellular factors such as particle surface, size, composition, and metal presence**. Manke et al. (2013)** discovered that through triggering associated cell signalling pathways, NP-induced oxidative stress responses served as warning signs for other negative effects such genotoxicity, inflammation, and fibrosis. It is critical to describe the ROS response triggered by NP since oxidative stress plays a significant role in the harm that this drug causes. To build a systemic toxicity screen with oxidative stress acting as a predictive model for NP-induced damage, it is critical to characterise the NP's physicochemical properties and to understand the several signalling cascades that are started by NP-induced ROS.

This is in line with the findings of **Abou-Zeid et al., 2021** who administered rats with or without oral sesamol (100 mg/kg) for 28 days after gavageing AlNPs (100 mg/kg) to the rats. MDA and 8-OHdG readings that are higher than normal signify oxidative DNA damage and lipid peroxidation, respectively.

Yousef et al. (2019) provided evidence supporting our findings by showing the detrimental effects of Al2O3NPs and ZnONPs individually and/or in combination on the testicular architecture. This led to difficulties with a variety of pathways important for mitochondrial biogenesis and function, as well as the induction of oxidative stress, lipid peroxidation, nitric oxide production, and pathways for necrotic and

apoptotic cell death in the testicular tissues. Other contributing factors include imbalanced sex hormones, histopathological problems, increased levels of tumour necrosis factor and interleukin 6, and activation of the inflammatory system.

In the striatum, cerebral cortex, olfactory bulb, and hippocampal regions of the brain, nano-Al2O3 accumulated, causing ultrastructural changes, oxidative damage, inflammatory reactions, and histopathological damage, according to **Liu et al., 2020.** Adding nano-Al2O3 reduced the number of live cells while raising lactate dehydrogenase and oxidative stress, according to in vitro testing.

Mitochondrial toxicity markers were evaluated between the two types of aluminium by **Arab-Nozari et al. in 2019.** They demonstrated that, due to AlNPs' increased reactivity, their harmful effect on isolated brain mitochondria was significantly greater than that induced by aluminium ions at various doses.

Rajivet et al. (2016) used in vitro toxicity assays to pinpoint the dangerous NPs. Contrary to our findings, this work primarily focuses on the comparative in vitro cytotoxicity and genotoxicity of four distinct NPs on human cells, including cobalt (II, III) oxide (Co3O4), iron (III) oxide (Fe2O3), silicon dioxide (SiO2), and aluminium oxide (Al2O3).

Based on the results of our research, microcurcumin can be used to protect against neurotoxicity caused by things other than aluminum.

In 2021, **Khadrawy et al.** published a study on the protective effects of nanocurcumin against the neurotoxicity brought on by doxorubicin in rat brain tissue. The findings of our study were consistent with their findings. They examined the neurochemical alterations brought on by an acute dosage of DOX (20 mg/kg, i.p.), and they assessed nanocurcumin's (NC) neuroprotective abilities (50 mg/kg, p.o.). They got to the conclusion that NC protection stopped the DOX-induced oxidative stress from modifying its parameters. Several of these negative effects were reduced by protection with NC, making DOX more acceptable. The preventive benefits of curcumin and nanocurcumin on CuSO4-induced brain oxidative stress, inflammation, and apoptosis in rats were studied by **Sarawi et al. in 2021.** In contrast to a decline in GSH, SOD, and catalase levels, malondialdehyde (MDA) levels rose substantially in the brains of rats given copper treatment.

We discovered that it is crucial to discover a quantifiable marker to identify who is susceptible to the presence of severe brain toxicity, and we want to apply it in the future to children in both high and low aluminium schedules in the United States and Scandinavian countries. Fortunately, we applied the critical threshold methods to separate the high-level Al schedule from the control group and the low-level Al schedule from the control group and, thankfully, found cut off values. Our results include cutoff values for iNOS and Bax. Those values are 215 and 216.5, respectively, that can discriminate between the "high Al" schedule and the control group with sensitivity = 100% and specificity = 100%. Values that exceed the cutoff are assigned to the "high Al" schedule.

On the other hand, Bcl2 cannot be used for discrimination between both groups. To discriminate between the "low A1" schedule group and the control group. The cutoff values for iNOS and Bax are 200 and 202, respectively, that can discriminate "low A1" schedules from the control group with sensitivity = 100% and specificity = 100%. Group 2 includes values above the cutoff. On the other hand, Bcl2 cannot be used for discrimination between group 2 and group 5.

Our study's findings confirm **Danielsson et al's (2018)** assessment of the hypothesis on the immunostimulatory effects of aluminium adjuvants, which focused on how cellular stress resulted in an inflammatory response. They discovered that a protracted intracellular buildup of aluminium adjuvants serves as a solid store for sparingly soluble aluminium salts, maintaining a consistent level of Al3+ ions in the cytoplasm and affecting a number of metabolic processes.

According to Danielsson et al., persistent stress will induce cells to activate Danger Associated Molecular Patterns (DAMP) signalling (2018). They also suggested that one key element of the immune-stimulating properties of aluminium adjuvants is the maintenance of a constant concentration of Al3+ ions in the cytoplasm.

In contrast to what we found, **Liang et al. (2021)** addressed this problem and investigated the precise mechanism of adjuvanticity at the nano-bio interface by developing a library of amorphous aluminium

hydroxyphosphate nanoparticles (AAHPs) with distinct surface features. Positively charged particles greatly outperform neutral or negatively charged counterparts when it comes to inducing an immunological response, suggesting that AAHPs are capable of disrupting cell membranes and causing inflammatory reactions. Staphylococcus aureus (S. aureus) recombinant protein is used in a vaccine that improves humoral immunity and prevents S. aureus sepsis in mice models.

Additionally, **Liang et al. (2021)** demonstrated that serum antigen-specific antibodies are more likely to be produced when particles with positive charges are combined with particles that mimic the human papillomavirus type 18 virus. This study shown that AAHPs with well-controlled physicochemical features can be produced. This is crucial for establishing a structure-activity link, which will help direct the creation of tailored nanomaterial-based adjuvants that may be incorporated into vaccinations to safeguard human health.

Conclusion:

Based on all our findings, we concluded that the use of nano-aluminium adjuvants in our vaccination schedules poses many risks to the health of our children. It is produced by histopathological alterations in brain tissue, a rise in oxidative indicators, and a decrease in antioxidants. Nanocurcumin can cause marked improvements in neurotoxicity, apoptosis-producing monoclonal antibodies, oxidative stress caused by nanoaluminum adjuvants due to its high antioxidant properties, and histopathological changes caused by nanoaluminum adjuvants. Alternatives to aluminium adjuvants for stimulating immunity by vaccines have been discovered, according to the current study. If it is a must, the dose should be decreased. Furthermore, we need to conduct additional studies to find a solution to this problem, particularly given the rising incidence of neurological disorders among our young people.

Acknowledgement:

None

Declarations of interest:

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus, membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Data availability statement:

The data that support the findings of this study are available on request from the corresponding author.

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