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Histopathological study of zinc oxide nanoparticle-induced neurotoxicity in rats

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ABSTRACT

Zinc oxide nanoparticle is considered to be one of the most important nanoparticles because of its wide range of applications in many industries such as electronics, cosmetics, personal care products and food additives. As a result of its widespread use, humans become more exposed to zinc oxide nanoparticles *via* ingestion, inhalation, and dermal penetration. This study aims to investigate the toxic effect of zinc oxide nanoparticles on the brain and spinal cord in rats by the assessment of histopathological changes using light and electron microscopic examination. Eighty adult albino rats were divided into four groups, each group comprising twenty rats. The control group received water *via* oral gastric tube while the second, third and the fourth groups received 50, 200 and 400 mg/kg/day of zinc oxide nanoparticles, respectively *via* intraperitoneal injections for two months. Zinc oxide nanoparticles led to degeneration in the brain and spinal cord including pyramidal and glial cells, white and gray matter associated with pyknotic nuclei and disturbance in many cytoplasmic organelles. Prolonged use of zinc oxide nanoparticles induced ultrastructural and histopathological changes in brain and spinal cord depending on its dose.

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1. INTRODUCTION

Nanoparticles are small objects that are less than 100 nm in size with a large specific surface area and high particle number per unit mass, and hence they can cause different degrees of biological effects which may affect the health safety [1]. Furthermore, nanoparticles have widespread application in biomedicine and many industries because they have unique thermal, mechanical, magnetic and optical properties [2].

In recent years, many published articles have showed that nanoparticles have a greater toxicity in comparison to the large-size materials because they are highly reactive causing oxidative stress in humans and animals. They reach into the circulation and can pass easily through the cell membrane and the blood-brain barrier leading to inflammatory, oxidative and cytotoxic effects [3].

Zinc is considered to be one of the nutritional supplements and food additives; it has a broad range of applications in many industries such as dyes, paints, pigments, rubber, electronics, cosmetics and the personal care products [4]. Zinc oxide nanoparticle is generated from the melting and oxidizing of zinc at a high temperature. It is commonly used in medicines because of its

antibacterial effect; it affects the bacterial growth of *staphylococcus*, *streptococcus*, and *E. coli* preventing the spread of infection. As a result of its widespread use, humans become more exposed to zinc oxide nanoparticles *via* many routes such as ingestion, inhalation, intravenous injection and dermal penetration [5].

Although numerous studies have investigated nanoparticle toxicity, showing the cell response based on the nature and the dose of nanoparticles, and its dangerous effects on human health, these studies did not investigate the toxicity of zinc oxide nanoparticles on some critical systems such as neurological system [6].

Therefore, the current study aims to investigate the toxic effect of zinc oxide nanoparticles on the brain and spinal cord in rats *via* the assessment of histopathological changes using light and electron microscopic examination.

2. MATERIALS AND METHODS

Eighty healthy male adult albino rats aged about 14-16 weeks (200-300 g) were maintained on a 12-h. light/dark cycle. They were fed water and standard rat pellets during the experimental period. The rats were divided into four groups, each group comprising twenty rats. The first group (control) received 0.5 ml of distilled water only *via* an oral gastric tube while the second, third and the fourth groups received 50, 200 and 400 mg/kg/day of zinc oxide nanoparticles, respectively *via* intraperitoneal injections [7, 8]. The daily administration of distilled water and zinc oxide nanoparticles continued for two months.

The dispersion of zinc oxide nanoparticles was less than 100 nm where the particle size (DLS) was less than 35 nm avg. part. size (APS). The concentration was 50 wt.% in H_2O while the pH was 7 $\pm$ 0.1 for aqueous systems (Lot/No. MKBN3534V-Nano - Sunguard^{TM} in water). The zinc oxide nanoparticles were manufactured by Sigma-Aldrich Co., St. Louis, USA [9].

2.1. Histopathological studies

Twenty-four hours after the last administration of zinc oxide nanoparticles, the rats were excessively anaesthetized *via* intraperitoneal injections using a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg). Transcardial perfusion for central nervous system fixation was carried out using butterfly needle in the left ventricle of the rat heart to pass into the ascending aorta. Initially, intravascular perfusion was done using 0.9% saline solution to clear the blood from the body, and then it was changed to 4% paraformaldehyde in phosphate buffered saline solution. After the completion of the perfusion process, the tissues were dissected from the cerebral cortex, brain stem and spinal cord segments (C4 - T6 - L2). They were trimmed and fixed in 4% paraformaldehyde for 24 hours at 4 °C and then washed and stored in phosphate buffered saline [10, 11]. They were dehydrated in ascending grades of alcohol, cleared in xylene, embedded in paraffin, sectioned at 4-6 μm thickness and stained with haematoxylin and eosin [12].

Ultrastructure studies were performed using the transmission electron microscope. The tissue specimens of the brain and spinal cord were prepared by soaking and fixating the specimens in 2.7% glutaraldehyde solution in 0.1 M phosphate buffer for 1.5 hours at 4 ºC. They were then washed in 0.15 M phosphate buffer (pH 7.2) and post-fixed in 2% osmic acid solution in 0.15 M phosphate buffer for one hour at 4 ºC. Dehydration was done using acetone. They were then embedded in Epon resin 812. Tissue sections were cut at 70 nm thickness using an ultramicrotome LKB. Ultrathin sections were stained with uranyl acetate and lead citrate. Tissue sections were evaluated using transmission electron microscope [13].

2.2. Ethical considerations

The most appropriate animal species were chosen for this research. Promotion of high standard care and animal well-being were exercised at all times. An appropriate sample size of animals for the experiment was calculated using the fewest number of animals to obtain valid results statistically. Painful procedures were performed under anaesthesia to avoid any distress and pain that could be inflicted on the animals. The Institutional Animal Ethics Committee approved our standards of animal care and administration that are consistent with the requirements and standards of international laws and regulations.

3. RESULTS

3.1. Histopathological findings using a light microscope

3.1.1. Brain

Examination of brain sections in the control group rats showed a normal structure (Fig. 1A). But, the brain tissues of the second group rats which received 50 mg/kg/day of zinc oxide nanoparticles showed a congestion in the blood vessels, and degeneration in some pyramidal and glial cells (Fig. 1B). The brain of the third group rats which received 200 mg/kg/day of zinc oxide nanoparticles showed a mild degeneration of the pyramidal and glial cells with small sized vacuoles (Fig. 1C). In the fourth group rats which received 400 mg/kg/day of zinc oxide nanoparticles, the brain tissues showed a loss of standard architecture, widespread degeneration of pyramidal and glial cells, increased

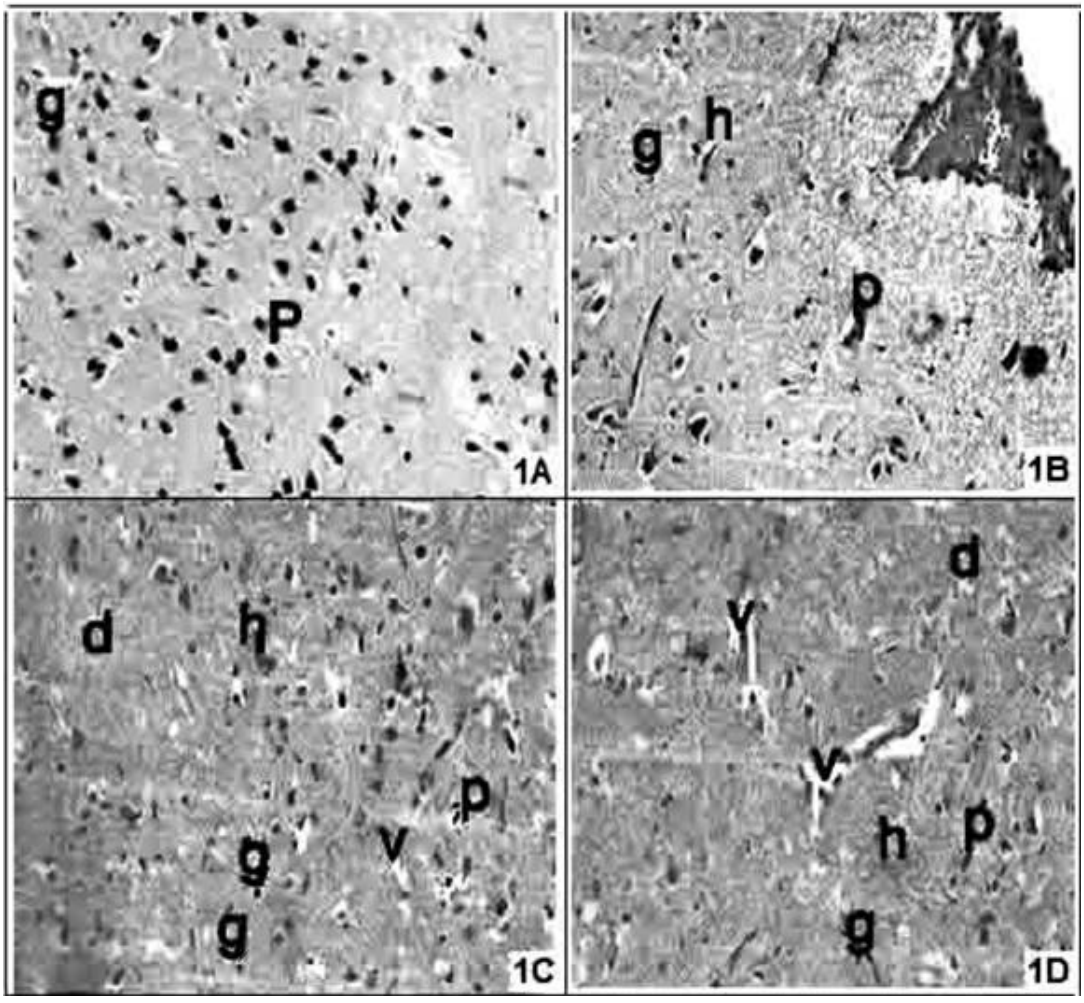

Fig. 1. (1A) A photomicrograph of a section of brain of the control group rat showing the standard structure of white matter, gray matter, pyramidal (p), and glial cells (g); **(1B)** a photomicrograph of a section of brain of the second group rat showing the congested blood vessels (h), some degenerated pyramidal (p), and glial cells (g); **(1C)** a photomicrograph of a section of brain of the third group rat showing the congested blood vessels (h), small sized vacuoles (v) and degenerated areas (d), mild degenerated pyramidal (p) and glial cells (g); **(1D)** a photomicrograph of a section of brain of the fourth group rats showing widespread degenerated areas (d), an increasing number of fully congested blood vessels (h), large sized vacuoles (v), shrinkage and degeneration of pyramidal (p) and glial cells (g) (H&E X 400).

number of fully congested blood vessels, pyknotic nuclei and increased size of vacuoles (Fig. 1D).

3.1.2. Spinal cord

The examination of spinal cord tissues in the control group rats showed normal histological structure (Fig. 2A). The spinal cord tissues of second group rats which received 50 mg/kg/day of zinc oxide nanoparticles showed a mild degeneration in the white and gray matter along with the appearance of mild vacuoles, degenerated nuclei in some cells, pyknotic nuclei in some others and fully congested blood vessels (Fig. 2B). The spinal cord tissues of third group rats which received 200 mg/kg/day of zinc oxide nanoparticles

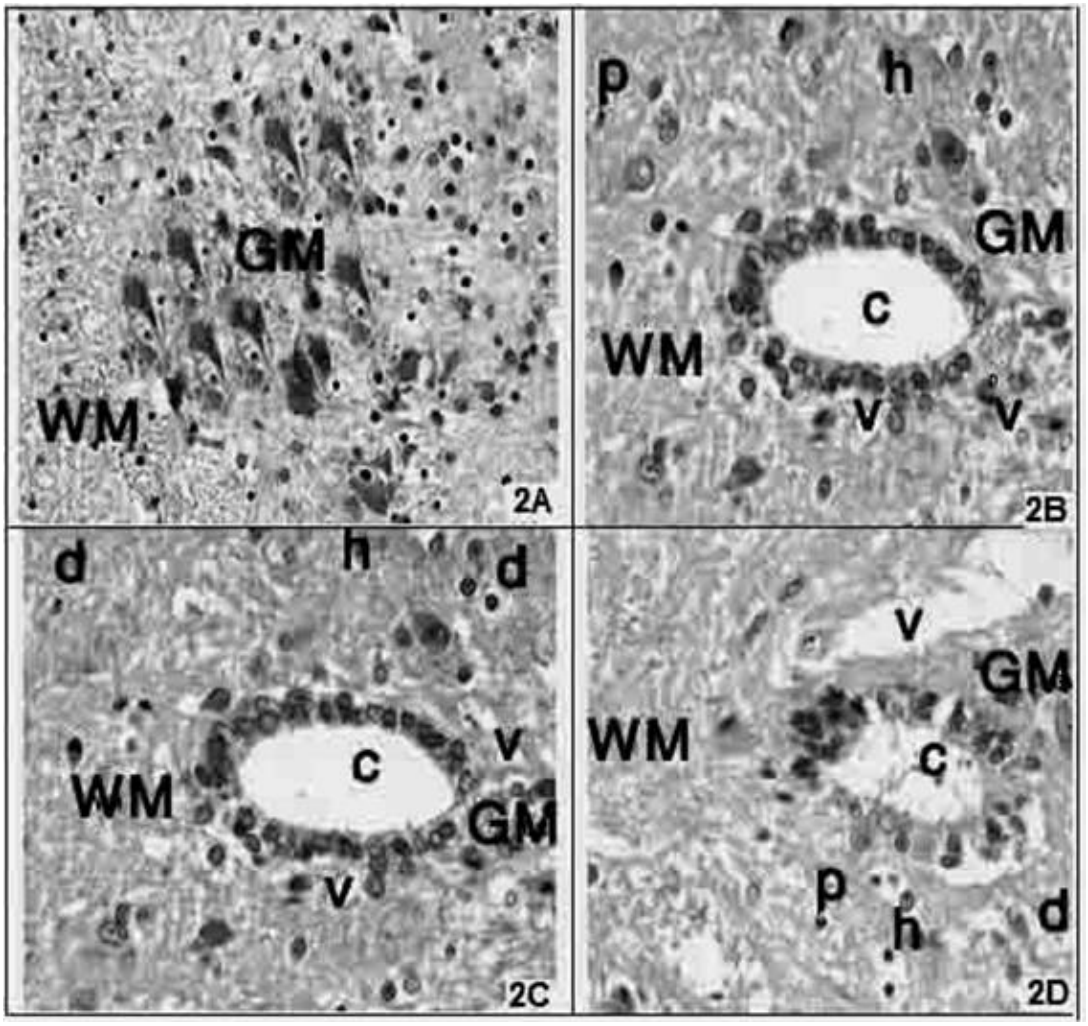

Fig. 2. (2A) A photomicrograph of a section of spinal cord of the control group rat showing the conventional structure of white matter (WM) and gray matter (GM); **(2B)** a photomicrograph of a section of spinal cord of the second group rat showing the central canal (c), fully congested blood vessels (h), and pyknotic nuclei (p). There are also vacuoles (v) in the outer white matter (WM) as well as in the internal gray matter (GM); **(2C)** a photomicrograph of a section of spinal cord of the third group rat showing disintegration in the ependymal cells of the central canal (c), moderate degeneration in the cells of outer white matter (WM) and inner gray matter (GM). There are also fully congested blood vessels (h), the small sized vacuoles (v) and the degenerated areas (d); **(2D)** a photomicrograph of a section of spinal cord of the fourth group rat showing severely degenerated cells in the outer white matter (WM) and internal gray matter (GM). There are disintegrated ependymal cells of the central canal (c), extensive congestion in the blood vessels (h) with huge-sized vacuoles (v) and pyknotic nuclei (p) (H&E X 400).

showed a moderate degeneration in the outer white matter and inner gray matter, and in the ependymal cells of the central canal with sporadic small vacuoles (Fig. 2C). The spinal cord tissues of fourth group rats which received 400 mg/kg/day of zinc oxide nanoparticles showed a severe degeneration in the central canal ependymal cells, white and gray matter. Moreover, there were pyknotic nuclei as well with a lot of degenerated nuclei in some cells, and scattered large vacuoles in the white and gray matter (Fig. 2D).

3.2. Ultrastructural findings using an electron microscope

3.2.1. Brain

The ultrastructure of brain cells in the control group rats showed normal appearance (Fig. 3A). The ultrastructure of brain cells in the second group rats showed scanty cytoplasm containing a few cisternae of rough endoplasmic reticulum and mitochondria, rounded indented euchromatic nuclei with prominent nucleoli, a few number of apoptotic cells with heterochromatic shrunken nuclei and dense cytoplasm (Fig. 3B). The ultrastructure of brain cells in the third group rats showed a small number of healthy cells with euchromatic nuclei and scanty cytoplasm containing a few organelles, an increase in the number of shrunken nuclei in the degenerated neuronal cells (Fig. 3C). The ultrastructure of brain cells in the fourth group rats also showed a few numbers of healthy cells with euchromatic nuclei and scanty dark cytoplasm containing cisternae of rough endoplasmic reticulum, degenerated mitochondria and wide vacuoles, an increase in the number of shrunken and degenerated neuronal cells with pyknotic nuclei (Fig. 3D).

3.2.2. Spinal cord

The ultrastructure of spinal cord cells in the control group rats showed normal appearance (Fig. 4A). The ultrastructure of spinal cord cells in the second group rats showed scanty cytoplasm containing a few ribosomes and a huge number of mitochondria, revealed disrupted mitochondrial cristae with dilated oligodendrocytes, a few number of apoptotic degenerative cells with condensed heterochromatic shrunken nuclei and dense cytoplasm (Fig. 4B). The ultrastructure of spinal cord cells in the third group rats showed an increase in the shrunken and degenerative neuronal cells number with pyknotic nuclei (Fig. 4C). The ultrastructure of spinal cord cells in the fourth group rats also showed shrunken and degenerative neuronal cells with condensed heterochromatin in the nucleus and disrupted nuclear membrane. Furthermore, there was empty degenerative cytoplasm with dispersed residual organelles such as fragmented rough endoplasmic reticulum, degenerative mitochondria, and disrupted cristae with vacuoles, swollen myelinated and unmyelinated axons (Fig. 4D).

4. DISCUSSION

Zinc oxide nanoparticles are increasingly utilized in different industrial and medical applications where the various nanoparticles with different physicochemical characters have different toxicological effects. Hence, it is highly important that we study their toxic effects on various body systems, especially neurological system. The current study investigates the toxic effect of zinc oxide nanoparticles on the brain and spinal cord tissues in rats using the light and electron microscope examination.

Our results revealed many histopathological and ultrastructural changes in the brain and spinal cord depending on the dose of zinc oxide nanoparticles; they range from congestion and mild degeneration to severe degeneration associated with ultrastructural disturbance in the neuronal cells and apoptotic degenerative cells. These results are consistent with Migliore and colleagues [14] who reported that zinc oxide nanoparticles cause neurodegeneration in the brain tissues, and in agreement with Win-Shwe and Fujimaki [15] who showed that the toxic effects of zinc oxide nanoparticles on the central nervous system include cytotoxicity, inflammation, and oxidative stress induction that result in neurodegeneration.

According to [16], the mechanism of zinc oxide nanoparticle toxicity depends on the oxidative stress generation that causes an inflammation process based on the activation of inflammation-related genes. In the same context, it has been shown that the oxidative stress of zinc oxide nanoparticles have the ability to affect the integrity

and permeability of blood-brain barrier *via* inducing endothelial cell leakiness and pro-inflammatory mediators, which is in agreement with [17, 18]. Neurotoxicity may also occur *via* a direct effect on the brain itself or by an indirect systemic inflammatory effect. In the contrasting context, Zheng, Y. *et al*. [19] confirmed that zinc oxide nanoparticles don't have any toxic effect on the brain tissues, which is in agreement with [20].

The brain is considered to be the most vulnerable organ to the oxidative stress because of its low level of antioxidants, its high energy demand, and the high cellular content of lipids and proteins.

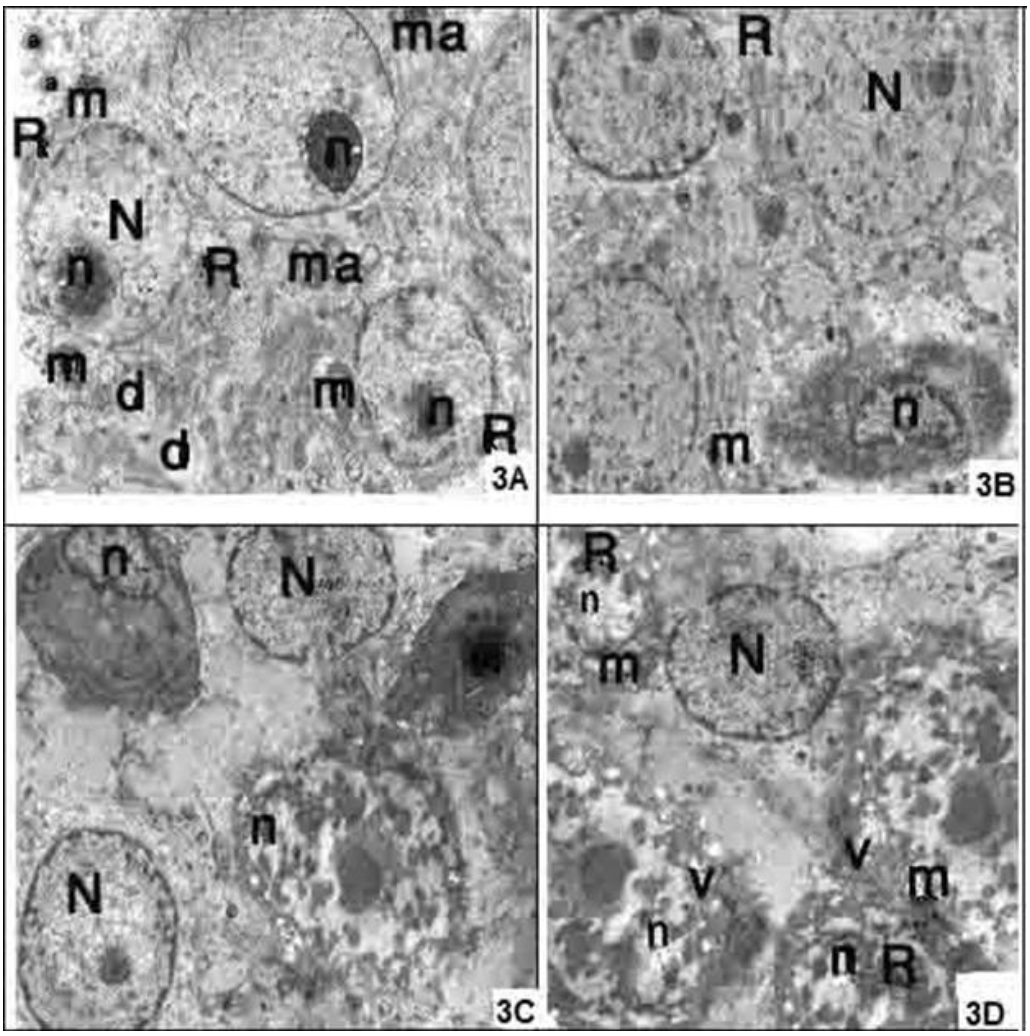

Fig. 3. (3A) Electron microscopic picture of the brain cells of the control group rat showing euchromatic nuclei (N) with prominent nucleoli (n), mitochondria (m) and rough endoplasmic reticulum (R), dendrites (d), unmyelinated (a) and myelinated axons (ma); **(3B)** electron microscopic picture of the brain cells of the second group rat showing euchromatic nucleus (N), scanty dense cytoplasm (n) containing a few organelles such as mitochondria (m) and ribosomes (R), apoptotic neuronal cells with the heterochromatic shrunken nucleus; **(3C)** electron microscopic picture of the brain cells of the third group rat showing euchromatic nucleus (N), scanty dense cytoplasm (n) containing a few organelles with an increase in the number of shrunken nuclei; **(3D)** electron microscopic picture of the brain cells of the fourth group rat showing euchromatic nucleus (N) and scanty dark cytoplasm containing a few degenerated mitochondria (m). It also shows pyknotic nuclei (n), the dilated cisternae of rough endoplasmic reticulum (r), wide vacuoles (v), and an increase in the number of shrunken and degenerated neuronal cells (X8000).

The microglial activation produces high levels of free radicals that can damage proteins, lipids, and nucleic acids at the particle deposition site [21].

Zinc oxide nanoparticles induce upregulation of inflammation, apoptosis and oxidative stress pathways in the spinal cord cells with downregulation of energy metabolism *via* the release of different

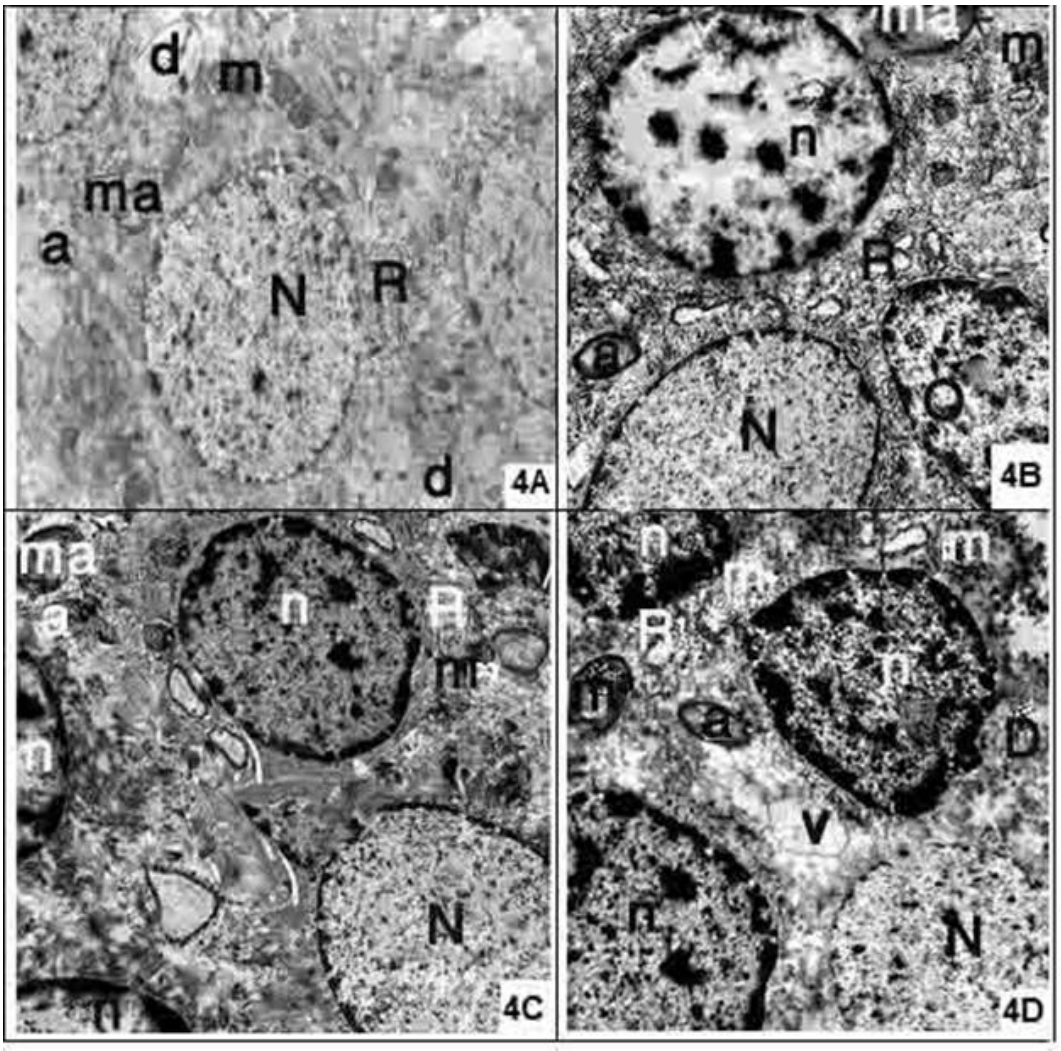

Fig. 4. (4A) Electron microscopic picture of the spinal cord cells of the control group rat showing heterochromatic nuclei (N), mitochondria (m) and rough endoplasmic reticulum (R), dendrites (d), unmyelinated (a) and myelinated axons (m); **(4B)** electron microscopic picture of spinal cord cells of the second group rat showing apoptotic cells with the heterochromatic shrunken nucleus (N) and dense cytoplasm (n). It also shows swollen mitochondria (m) and ribosomes (R), unmyelinated (a) and myelinated axons (ma), dendrites (d) and the dilated oligodendrocyte (O); **(4C)** electron microscopic picture of the spinal cord cells of the third group rat showing the heterochromatic nucleus (N) and the degenerated neuronal cells with pyknotic nuclei (n), dilated cisternae of the degenerated mitochondria (m) and rough endoplasmic reticulum (R), and the myelinated (ma) and unmyelinated axons (a); **(4D)** electron microscopic picture of the spinal cord cells of the fourth group rat showing a few healthy neuronal cells with the heterochromatic nucleus (N). It also shows the shrunken and degenerated neuronal cells and the condensed heterochromatin in the nucleus (n) with the degenerated mitochondria (m). There are empty and degenerated cytoplasm (D), the fragmented rough endoplasmic reticulum (R), the disrupted cristae with vacuoles (v), and the swollen myelinated (ma) and unmyelinated axons (a) (8000X).

mediators such as toxic mediators (excitatory neurotransmitters) or anti-toxic mediators such as anti-inflammatory cytokines and neurotrophins to cause neurodegeneration [22].

Valdiglesias, V. *et al*. [23] showed that the neurotoxicity effect of zinc oxide nanoparticles is dose-dependent, which is in agreement with our results. In the related context, Yin, Y. *et al*. [24] also showed that zinc oxide nanoparticles cause alterations in the cell cycle and mitotic arrest in the neuronal cells inducing an increase in the apoptosis percentage in a mitochondria-independent pathway. Moreover, Fukui, H. *et al*. [25] found that the cytotoxicity of zinc oxide nanoparticles depends on the high level of zinc ions in mitochondria in correlation with the high levels of ROS, inducing an oxidative DNA damage, followed by cell death.

5. CONCLUSION

The prolonged use of zinc oxide nanoparticles may induce histopathological and ultrastructural changes in the brain and spinal cord depending on the dose and based on reactive oxygen species generation.

6. RECOMMENDATION

Further researches on humans using non-invasive magnetic resonance imaging (MRI) should be done in future to verify our results and confirm the neurotoxicity induced by prolonged use of zinc oxide nanoparticles, and to demonstrate the possible histopathological changes in the brain and spinal cord.

CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

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