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Selenium Protects Caspase 3 and Glial Fibrillar Acidic Protein (GFAP) Expression in Cerebellum of Rats against Zinc Oxid Nanoparticles (Znonps) Exposure

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ABSTRACT

Background: Growing evidences reported that zinc oxide nanoparticles (ZnONPs) could reach the brain after oral ingestion; however, the "neurotoxicity of" ZnONPs after oral exposure has not been fully investigated. This study aimed to confirm the "neurotoxicity of" ZnONPs and the possible neuroprotective role of selenium.

Material and Methods: Post acclimatization, forty male albino rat were categorized into four groups (n= 10); G1: Healthy control, G2: Selenium-administered rats, 0.2 mg/kg/day, oral gavage and G3-G4: Treated groups, orally- given 1 g/kg/day ZnONPs by gastric tube for 5 consecutive days and divided as follows; G3: ZnONPs intoxicated rats., G4: ZnO NPs intoxicated rats with co-administration of selenium daily. Selenium was orally-given for 8 consecutive days, 3 days of them before the start of the experiment.

Results: The exposure of experimental animals to ZnO NPs confirmed the induction of an elevated percentage of apoptosis in the cerebellar tissue leading to damaged neurocytes in the three cortical layers. This could be detected via a significant increase in the immunoreactivity of caspase 3(an apoptosis marker) and morphometric analysis due to the subsequent release of reactive oxygen species (ROS. Concerning GFAP, a significant decrease in the immunoreactivity in astrocytes and glial nerve fibers was recorded as compared to control. These observations may be due to a focal loss of cerebellar glial nerve cells or associated with astrocyte damage due to ZnO NPs intoxication and the possible change in their functional properties and disability to synthesize GFAP protein.

However, these measurements were improved after nutritional co- supplemntation of 0.2 mg/kg b.w. with ZnO NPs exposure.

Conclusion: The present results confirmed the "neurotoxicity of" ZnONPs after recurrent oral exposure via oxidative stress and apoptosis. However, supplementation of Se

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restricted and minimized this effect owing to its antioxidant properties recording a protective role.

Keywords: ZnONPs; brain; caspase-3; GFAP; albino rat.

INTRODUCTION

Nanotechnology is a rapidly developing science. Synthetic nanoparticles could be metal nanoparticles (e.g. gold and silver) or metal oxide nanoparticles (e.g. zinc oxide). Zinc oxide nanoparticles (ZnONPs) are widely used as a dietary supplement and food additive, which was applied in food packaging. Moreover, ZnONPs are commonly used in medicine for coating dental implants due to their good antifungal and antibacterial properties. Previous *in vivo* studies revealed that ZnONPs after absorption and systemic circulation could reach and induce cytotoxicity in different animal organs including lung, heart, kidney, liver, spleen, pancreas and testes [1–7]**.**The use of ZnO NPs became controversial since they can easily pass via cell membranes and interact with cellular macromolecules producing cytotoxic effects [Elshama *et al.*,8]. Due to the increasing use of metallic nanoparticles in medicine, attention is paid to the safety of using them for the central nervous system [Sawicki *et al.,* 9**].** Selenium (Se) is an essential trace element, required for the maintenance of growth and health [10]. It plays an indispensable role in illness prevention and fertility [11, 12]. There are relatively few reports on the biological significance of Se and it is still a big challenge for investigators to evaluate the potential health benefits of it. Astrocytes are the commonest cell type in the central nervous system (CNS) of vertebrates and glial fibrillary acidic protein (GFAP) is an essential component in these cells and its expression could be used as a marker of astrocyte maturation and differentiation. The study of GFAP regulatory genes may help in understanding both developmental signaling for astrocyte maturation and their response to injury of CNS [13, 14].

Therefore, the risk of toxicity due to the increasing exposure to ZnO NPs in our daily life and medicine is urgently needed to be fully-investigated. Accordingly, the aim of the current study is to investigate the effects of ZnO NPs in rats's cerebellum and the possible protective role of selenium via the immunoexpression of caspase-3 and GFAP.

2. Material and Methods

2.1. Chemicals

Zn O-NPs (< 50 nm size, MW: 81.39 g/mol, purity > 97% with long lasting effect) and selenium **(**Sodium selenite, Na_2SeO_3) were products of Sigma-Aldrich Corporation (USA). All other chemicals used in the study were of high analytical grade and products of Sigma and Merck companies.

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2.2. Animals

Forty adult healthy male albino rats weighing 180-200 g (10-12 week) of *Rattus norvegicus* strain were obtained from the animal house of Faculty of Veterinary Medicine, Zagazig University. Animals were survived in plastic cages under standard conditions of dark / light cycle, temperature (25°C ±1), good ventilation and humidity (55%). Free access to tap water and a standard diet was available to rats.

2.3. Experimental Design

Post acclimatization, animals were categorized into four groups (n= 10); G1: Healthy control, G2: Selenium-administered rats, 0.2 mg/kg bw/day, oral gavage [El-Demerdasha and Nasr, 15] and G3-G4: Treated groups, orally- given 1 g/kg b.w/day ZnONPs by gastric tube for 5 consecutive days [Wang *et al.*, 16 and Nassar *et al.*, 7] and divided as follows; G3: ZnO NPs intoxicated rats., G4: ZnO NPs intoxicated rats with co-administration of selenium daily. Selenium was orally-given (0.2 mg/kg bw /day) for eight consecutive days, 3 days of them before the start of the experiment. After 24 hours of the last dose administration, rats were fasted overnight, euthanized and sacrificed.

Ethics approval and consent to participate

State authorities approved the experiment and followed Egyptian animal-protection rules of IACUC at Zagazig University with approval number: ZU-IACUC/F/109/2020.

2.4. Tissue sampling

Brains were quickly excised from skulls after quick cervical decapitation, blotted out with filter paper. Brains were split sagitally into two hemispheres. Cerebella were harvested and fixed in 10% neutral buffered formalin at room temperature for immunohistochemical examination.

2.5. Immunohistochemical study

2.5.1. Caspase-3 immunostaining

Tissue sections were dewaxed, rehydrated and 10% H_2O_2 was added for endogenous peroxidase blocking. Then, boiling with citrate buffer (pH 6.0) for antigen retrieval was performed. Sections were then incubated with the primary antibodies: anti-caspase 3 (BIOCYC GmbH, Germany). Subsequently, incubation with the secondary antibody, a biotinylated goat anti-rabbit immunoglobulin and avidin–biotin complex was carried out.

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The localization sites for caspase-3 in the cerebellar tissue were visualized by adding diaminobenzidine HCl, which was converted into a brown precipitate by peroxidase. Finally, Mayer's hematoxylin was utilized for counterstaining [17].

2.5.2. GFAP immunostaining

Dewax and rehydrate tissue sections via ascending series of ethanol. Add 10% H_2O_2 for inhibition of endogenous peroxidase. Then, boiling with citrate buffer (pH 6.0) for antigen retrieval was done. Incubate with GFAP, mouse monoclonal antibody (1:500). Horseradish Peroxidase complex was used to amplify immunostaining (Dako, REALTM EnVision TM / HRP, Mouse ENV). Diaminobenzidine (3.3) was used to visualize sections. The substrate system developed a crisp brown end product at the sites of the target antigen. Counterstain with Mayer's haematoxylin, dehydrate, clear and cover with permount [18].

2.6. Image Analysis

Quantifcation of caspase-3 and GFAP was estimated by measuring the area % of expression from 5 randomly chosen fields in each section and averaged using image analysis software (on image pro plus 4.5.1 computer system). The system measured the area percentage of caspase-3 and GFAP positive expression.

2.7. Statistical analysis

The results were analyzed using the Statistical Package of Social Science (SPSS) version 10 software. Analysis of variance, one way (ANOVA) was used for comparison between groups.

RESULTS

3.1. Immunohistochemistry of Caspase- 3

In cerebellum of control rats, caspase-3(the executioner apoptotic protein) was rarely-expressed in the cytoplasm of granular, Purkinje and molecular neurocytes as a weakly-stained brown material (Fig.1) recording 15.99 % for positivity (Table 1, Fig. 5). Animals given selenium exhibited expression for casepase-3 in their cerebellar cells nearly similar to that of control group (Fig.2) recording 15.67 % for immunoreactivity (Table 1, Fig. 5). However, rats exposed to ZnONPs showed a high expression (24.96 %) for casepase-3 indicating increased apoptosis (Fig.3) and recording a significant increase ($p \leq 0.0001$) in its immunoreactivity as compared to that of control (Table 1, Fig. 5). Meanwhile, cerebellar sections of rats treated with selenium and exposed to ZnO-NPs showed a low

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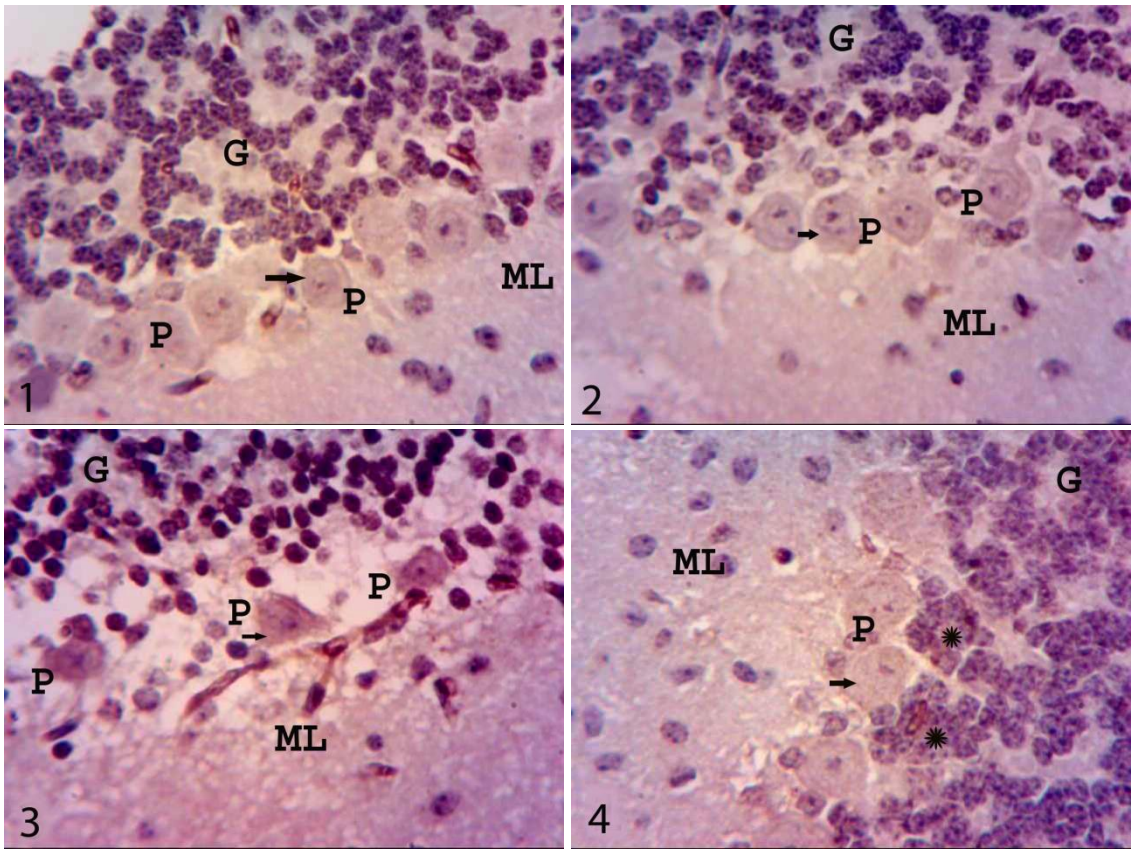
expression (19.95 %) recording a significant decrease ($p \leq 0.0001$) in the positivity of caspase-3 as compared to ZnONPs group (Table 1, Fig. 5).



Figs. 1-4: Sagital sections of cerebella of control and experimental rats (immunostained for caspase-3, X1000) exhibiting the expression of caspase-3 as brown deposited material in the cytoplasm of granular (G), Purkinje (P, arrows) and molecular (ML) cells. **Fig.1**: cerebellum of control rat revealing a weak immunoreactivity for caspase-3. **Fig. 2**: Cerebellum of selenium-administered rat exhibiting a caspase-3 expression nearly similar to that of the control. **Fig. 3**: Cerebellum of ZnONPs-exposed rat showing a strong reaction for caspase-3 in the cytoplasm of the apoptotic and damaged Purkinje cells. **Fig. 4**: Cerebellum of rats treated with selenium and exposed to ZnO-NPs revealing a decrease in the immunoexpression of caspase-3.

Table 1: Values of mean percentage area of caspase-3 expression in cerebella of control and treated rats.

	NC	Se	Zno NPs	ZnO NPs+ Se

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Mean %	15.99	15.67	24.96	19.95
Standard deviation	0.1043	0.0417	0.0611	0.0649
P Value		≤ 0.0003 Sig.	≤ 0.0001 Sig.	≤ 0.0001 Sig.

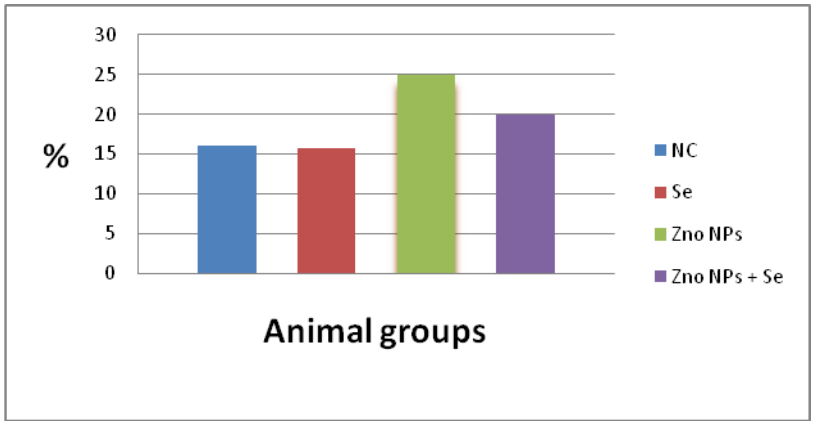

Fig. 5: Mean percentage area of caspase-3 expression in cerebella of control and treated rats.

3.2. Immunohistochemistry of GFAP

The immunohistochemical assessment of GFAP was performed followed by image and morphometric analysis to quantify the percentage of GFAP-positive constituents in the neural cells of cortical cerebellar layers in response to ZnONPs and selenium in different experimental groups (Figs.6-9). Cerebellum of control and selenium–given rats exhibited a high immunoreactivity for GFAP in different areas of gray and white matter (Figs.6, 7) to record 39.6% and 38.6 % respectively (Table 2, Fig. 10). The immunoreactive cells were large branched cells dispersed among various cell layers of cerebellum. However, weak GFAP expression could be detected in cerebellar tissue of ZnONPs-exposed rat (Fig. 8) showing less number and size of the GFAP immunoreactive cells and recording a comparable and significant decrease of positivity (28.1%) as compared to control (Table 2, Fig.10). Meanwhile, the combined administration of selenium and ZnONPs significantly increased GFAP expression (towards the normal status as compared to ZnONPs group (Fig.9) to record a percent of 36.6% (Table 2, Fig.10).

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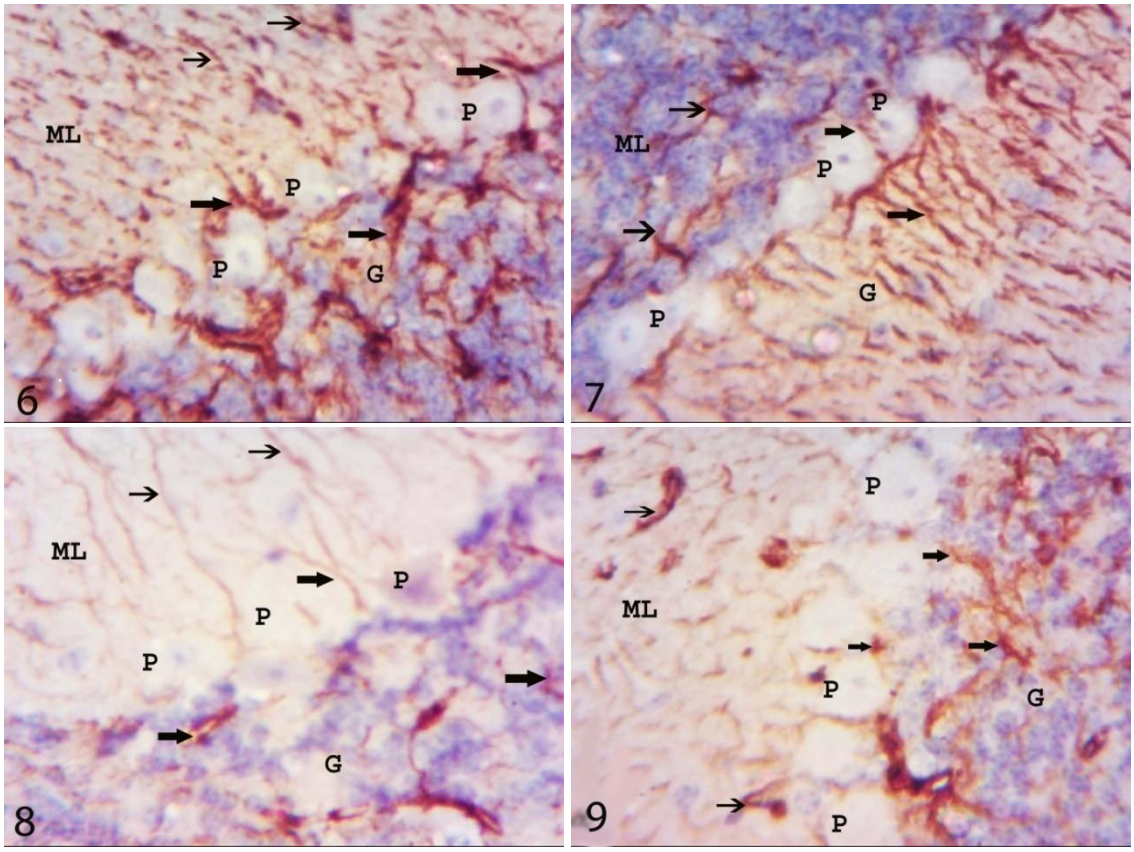

Figs. 6-9: Sagital sections of cerebella of control and experimental animals (GFAP immunostain, X1000) revealing immuno-expression for GFAP as a deep brown color in astrocytes of granular (G) and Purkinje (P) cell layers (thick arrows) and radial glial fibers of molecular layer (ML, thin arrows). **Fig.6**: Cerebellum of control animal, showing high expression of GFAP. **Fig.7**: Cerebellum of rat given selenium exhibiting a high GFAP immunoreactivity nearly similar to control. **Fig.8**: Cerebellar tissue of ZnONPs-exposed rat showing weak expression for GFAP in astrocytes and radial nerve fibers. Notice, the damaged Purkinje cells (p) and the low incidence of astrocytes in the three cortical layers. **Fig.9**: Section of cerebellar tissue of rat co-administered with ZnO-NPs and selenium showing increased GFAP positivity in astrocytes and radial nerve fibers.

Table 2: Values of mean percentage area of GFAP expression in cerebella of control and treated rats.

	NC	Se	ZnO NPs	ZnO NPs+ Se
Mean%	**39.6**	**38.6**	**28.1**	**36.6**
Standard deviation	**0.49**	**0.34**	**0.14**	**0.34**

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P Value		**≤ 0.002** **Sig.**	**≤ 0.0001** **Sig.**	**≤ 0.0001** **Sig.**

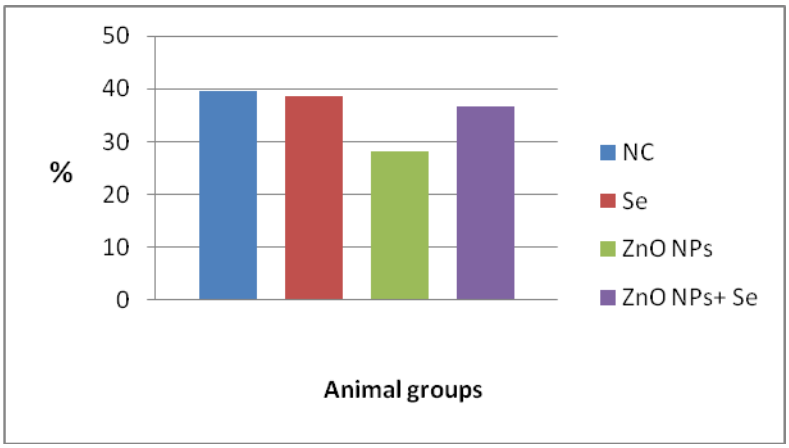

Fig.10: Percentage of GFAP immunoreactive cells in cerebella of different animal groups

DISCUSSION

Since nano materials constitute a large part of our daily life, the exposure to these substances becomes inescapable. Consequently, nanotoxicity is gaining great attention in later research work [19]. Most investigators focused their studies on the harmful effects of Zn ONPs on human health. Meanwhile, scarce data were available on the central nervous system. Hence, the target of the current work, cerebellum is a main nervous organ to be involved. The extensive use of ZnO NPs in food industry, medicine and agriculture enhances their contact with various organs and the subsequent cytotoxicity after oral exposure. Despite the unique beneficial properties of NPs, they may cause harmful effects due to their special characteristics including tiny sizes and large surface area for reactivity [20]. NPs may damage the blood brain barrier (BBB) causing increased permeability and BBB disruption and penetration of NPs leading to neurotoxicity [21,22]. Caspase-3 was chosen as a bio indicator in the current study since it is a key mediator of neuronal apoptosis, it is a common player involved and therapeutic target in neurodegeneration due to its involvement in neuro inflammation and apoptosis [23].

Caspase 3

As regards the impact of ZnO NPs on the activity of caspase-3; the exposure of experimental animals to ZnO NPs, in the present study, confirmed the induction of an

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elevated percentage of apoptosis in the cerebellar tissue leading to damaged neurocytes in the three cortical layers. This could be detected via the recorded significant increase in the immunoreactivity of caspase 3(an apoptosis marker) and morphometric analysis. However, these measurements were improved after nutritional co- supplementation of 0.2 mg/kg b.w. with ZnO NPs exposure.

These observations may be attributed to modification in the physicochemical properties of tissues and organs at a subcellular level and the subsequent release of reactive oxygen species (ROS) due to NPs exposure and penetration. These results are in agreement with previous studies of Khan et al**.** [23] who revealed that ZnO NPs injection significantly enhanced the area percentage of caspase 3 reaction which reflected an increase in the number of cells exhibiting positive reaction to caspase- 3 indicating apoptosis. Considering the toxicological pattern of ZnO NPs, Chang et al. **[**24**]** attributed it to the ROS which may mediate DNA single-stranded breaks. Other investigators revealed that ZnO NPs enter into brain areas systemic circulation to induce cytotoxicity in neuronal progenitor cells and neurons [25]. Also, Xiaoli et al. [26] recorded imbalanced antioxidant status and apoptotic death in brain cells of rat offspring after exposure to ZnONPs. Our results, also, are in harmony with those of other investigators; where Ibrahim et al. [27] recorded immunohistochemical results revealed that selenium NPs reduced the area percentage of caspase 3 reaction and the apoptotic index in hippocampal tissue of animals exposed to cyclophosphamide plus nano Se**).** Likewise, an in vitro study by Wang et al. [28] revealed that oxidative stress induced by ZnO NPs, activates apoptosis through triggering JNK signaling pathway in cultured primary astrocytes. Another study investigating the effect of nano Se on pancreatic cells, in a culture, and revealed that nano Se entirely inhibited the activation of caspase-3, 8, and 9 and accordingly inhibiting apoptosis [29]. Fluorometrically, Caspase 3/7 activity was analysed and measured by mapping in response to ZnO NPs. These NPs induced cellular apoptosis in astrocytes in a dose dependent manner and induced a statistically significant increase in caspase activity as compared to control [30]**.** Moreover, the oral exposure to ZnONPs (either 40 mg or 100 mg / kg daily for 7 days) induced brain tissue apoptosis as indicated by recorded DNA fragmentation and the elevation of caspase-3 (the executioner apoptotic protein) as well as increment of Fas (the extrinsic apoptotic protein) [31]**.** Some mechanisms may be implicated in ZnONPs-induced apoptosis. These include the liberation of ROS and the associated oxidative stress involving induction of lipid peroxidation in addition to the DNA damage [32-34]. Moreover, production of ROS may lead to the opening of mitochondrial membrane permeability transition pore and, accordingly initiate apoptotic signaling pathway [35]. Similarly; other studies provide evidences for the role of ROS as potential inducers of mitochondrial dysfunction and concomitant apoptotic cell death [36, 37].

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GFAP:

GFAP is a cytoskeletal protein expressed in astrocytes. It is a specific marker for the maturity of astrocytes. GFAP enhances the structural stability of astrocyte processes to help in changing astrocyte shape and mobility [38-40]. Astrocytes are glial cells involved in many cellular functions within CNS, including cell structure, movement, communication, and integrity of the blood brain barrier [41].

Considering the neural tissue of CNS in general and cerebellum in particular, astrocytes represent the most abundant glial cells. They carry out a verity of functions in CNS including axon conversion and synaptic support to BBB integrity and metal homeostasis. Astrocytes help in long term recovery during brain injury via surface molecule expression and release of trophic factor Blackburn et al., [42]. Due to these functional criteria, it is necessary to assess their toxic susceptibility as regards ZnO NPs exposure. Hence, GFAP is the marker of choice to reflect the immunohistochemical status of astroytes in response to ZnO NPs and selenium.

The immunohistochemical assessment of GFAP in cerebella of ZnO NPs - exposed animals, in the current study, recorded a significant decrease in the immunoreactivity in astrocytes and Glial nerve fibers as compaired to control. This was demonstrated and morphometrically analyzed in the form of less number and size of the GFAP - positive damaged astrocytes. These observations may be due to a focal loss of cerebellar glial nerve cells or associated with astrocyte consequent damage due to ZnO NPs intoxication and the possible change in their functional properties and disability to synthesize GFAP protein for playing their role in neuronal support in cerebellum at the conditions of the current experiment (dose: oral administration of 1g/kg /day of ZnO NPs for 5 days). Meanwhile, the combined administration of selenium and ZnO NPs significantly increased and restored GFAP expression towards the normal status as compared to ZnO NPs group. GFAP expression was increased to provide an evidence that astrocytes in their turn start to play their function in supporting and protecting the neighboring neurons. This improvement may be correlated to the scavenging effect of Se to the liberated free radicals. Antioxidant and anti-inflammatory effects of selenium were reported previously in vitro using cell lines and in vivo in animal models [43].

These protective results induced by nano Se were in harmony with the previous studies insuring the ameliorating effect of nano Se on the different tissues against the tissue deleterious effect. In this respect, Hamza et al.,2020 reported that nano Se could protect also against the hepatocellular damage induced by acrylamide [44]. Similarly, Abdel Hakeem et. al.,2020 reported that nano Se could mitigate the degeneration, necrosis, and inflammation of pancreatic acini and Langerhans cells caused by acute pancreatitis [45].

In contrast to these results, other investigators recorded increased expression of GFAP due to neuronal damage in midbrain of rats caused by the administration of acrylamide (30 mg/kg/day for 4 weeks) that might occur as a compensatory mechanism after neurodegeneration [46]. Previous studies considered the increased activation of astrocytes as a good indicator for reactive gliosis which occurs as a result of brain tissue damage [47]. This may interpreted that astrocytes could provide support and protection for neurons through cell-cell interaction [48].

Additionally**,** Ibrahim et al. [27] concluded that cyclophsphamide (CPH) triggered the activation of astrocytes that was manifested in the form of increased expression of GFAP. This might be explained as a compensatory mechanism for neuronal damage. The authors added, nano Se significantly reduced the expression of GFAB which means that nano Se could inhibit CPH induced neuronal damage and its concomitant astrogliosis. Moreover, Brain injury, whether to be chemical, traumatic or by a disease, mostly led to astrogliosis. The latter induces rapid synthesis of GFAP that occurs in situ and could be detected by immunostaining with GFAP antibodies [49].

Conclusion

Therefore, the present study effectively confirmed the neuroyoxic effects of ZnO NPs (1g/kg /day of ZnO NPs for 5 days) within the neuronal tissue of cerebellum of experimental animals and hence questions the safety facet of different application fields comprising ZnO NPs. It also added Se (0.2 mg / kg /day) provides a protective activity for the expression of caspase-3 and GFAP at the level of the used concentrations. A study which may be a useful resource for scientists working in the field of neurodegenerative diseases, oxidative brain injury or selenium-based drug development.

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