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Research paper

Selenium and zinc alleviate quaternary metal mixture -induced neurotoxicity in rats by inhibiting oxidative damage and modulating the expressions of NF-kB and Nrf2/Hmox-1 pathway

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ABSTRACT

Background: This study evaluated the potential protective effects of Zn and Se in the cerebellum and cerebral cortex, two fundamentally important brain regions, in albino rats that were exposed to heavy metals mixture (Al, Pb, Hg and Mn).

Methods: Animals were divided into five groups of seven animals per group with following patterns of exposure, controls group 1 were orally treated with deionized water for 60 days; group 2 was exposed to heavy metal mixture (HMM) with following concentrations (20 $mg{\cdot}kg^{-1}$ of Pb body weight; 0.40 $mg{\cdot}kg^{-1}$ of Hg; 0.56 $mg{\cdot}kg^{-1}$ of Mn; and 35 $mg{\cdot}kg^{-1}$; of Al), while groups 3,4 and 5 were exposed to HMM and orally co-treated with zinc chloride ($ZnCl_2$; 0.80 mg/kg), sodium selenite (Na_2SeO_3;1.50 mg/kg) and zinc chloride plus sodium selenite ($ZnCl_{0.2} + Na_2SeO_3$) respectively.

Results: Exposure to HMM depressed cellular antioxidant apparatus, induced generation of lipid peroxidation markers (Malondialdehyde and NO), downregulated expression of transcription factors (Nrf2, and NF-kB) and upregulated Caspase 3 levels. HMM potentiated acetylcholinesterase activity and induced moderate histopathological alterations. Nevertheless, Zn, Se and in particular Zn + Se had recovering effects on all mentioned hazardous effects produced by HMM exposure in the cerebral cortex and cerebellum.

Conclusions: Selenium and zinc exert neuroprotection via Nrf2/NF-kB signaling pathways against quaternary heavy metal mixture-induced impairments in albino Sprague Dawley rats.

Introduction

The end of the twentieth and the beginning of the twenty-first century brought a great step forward in the exploitation of various metal ores and in the humankind development as well. Nevertheless, the price which appeared as natural outcome of anthropogenic emissions is that human environment became polluted with the various metals such as aluminum (Alasfar and Isaifan, 2021), mercury (Xu et al., 2015, Rahman and Singh, 2019) and lead (Rahman and Singh, 2019). Gastrointestinal tract is the main route of heavy metals body entrance, however even in never smokers, air, which is highly polluted in urban areas, represents a great risk for health mainly when rich in the particulate matter 2.5 (PM 2.5); namely, some metals such as aluminum, lead and mercury are found bound to PM 2.5 (Hassanvand et al., 2015, Kermani et al., 2021) and PM 2.5 is particularly health hazard because of their ability to overcome all the impediments in respiratory tract and enter a bloodstream. On the other side, individuals who smoke have increased levels of lead in the blood (Repić et al., 2020, Shakeri et al., 2021). So, it became inevitable to get in contact with either aluminum, lead or mercury during the daily routine in majority of persons, and all of them are highly toxic to humans. In 2019, Agency for Toxic Substances and Disease Registry published list of substances which pose the most significant potential threat to human health; lead and mercury occupied second and third positions respectively ahead of cadmium and vinyl

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chloride and benzene.

In vivo studies showed that aluminum is toxic to various regions in the central nervous system such as hippocampus (Fernandes et al., 2020, Bittencourt and Damasceno-Silva, 2022), and pre-frontal cortex (Fernandes et al., 2020). Moreover, results of the study which involved human infants indicated that, aluminum is able to induce impairments in the motor development in dose-dependent manner (Ma et al., 2021), and cerebral cortex as well as cerebellum are fundamental for the normal performance of each movement. On the other side, exposure to lead is linked to cognitive impairment and social behavior, as well as with fine motor control disturbances (Ramírez Ortega and González Esquivel, 2021). Also, low-level methylmercury exposure may induce behavioral impairments (Martins et al., 2021). Manganese, which is physiological elements, is necessary for activation of many enzymes in metabolism, could exert its neurotoxicity when accumulate in excess via several mechanisms mediated by the NF-kB signaling pathway (Martins et al., 2021). Moreover, NF-kB signaling pathway could be involved in the pathogenesis of various neurodegenerative disorders such Alzheimer's Disease (AD) (Sun et al., 2022); aluminum exposure has been also linked with onset of AD (Colomina and Peris-Sampedro, 2017). Mercury is known to be neurotropic, also it has been shown that aluminum accumulates in all lobes of cerebral cortex and in the cerebellum. Majority of the mentioned functions such as behavior and motor skills are localized in the cerebral cortex; cerebellum is also important part of the motor system and further examination of heavy metals induced neurotoxicity in the cerebellum and cerebral cortex would be beneficial.

The long period of crude oil exploration and artisanal refining of crude oil in Niger delta, Nigeria have impacted negatively and added to the environmental burden of heavy metals (Pb, Hg, Al and Mn) with attendant public health issues (Okoye et al., 2021). Previously our lab has confirmed the presence of these metals lead (Pb), mercury (Hg), manganese (Mn) and aluminum (Al), among others in foods and beverages (Maduabuchi et al., 2008, Roberts and Orisakwe 2011). Furthermore, the metals of interest in this study have been predominant in electronic waste (e-waste), which has negatively impact on environment in Nigeria (Orisakwe et al., 2019, Frazzoli et al., 2022).

There is still limited data on the of heavy metals mixture (HMM) induced neurotoxicity and cerebral cortex and cerebellum are important CNS regions; also, zinc and selenium are important for cellular metabolism on one side and, they are capable to revert kidney damage produced by some heavy metals such as cadmium; therefore the aim of this study is to investigate whether zinc and selenium may revert neuronal damage produced by HMM exposure (Al, Pb, Mn and Hg) in the cerebral cortex and cerebellum.

Materials and methods

Chemicals

Lead acetate [$Pb(C_2H_3O_2)_2$], Aluminum chloride ($AlCl_3$), Mercury Chloride ($HgCl_2$) and Manganese dichloride ($MnCl_2$) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Inflammatory cytokines (tumor necrosis factor alpha; TNF – α, interleukin 6; IL – 6), transcription factors and apoptotic marker [(nuclear factor kappa B; NF-kB), (heme oxygenase-1; Hmox-1), (nuclear factor erythroid 2–related factor 2; Nrf2) and (Caspase 3)] ELISA Kit (for rats) were purchased from Elabscience science Biotechnology Company, (Beijing, China). All other reagents were of analytical grade and were obtained from the British Drug Houses (Poole, Dorset, UK).

Animals and treatments

Female Sprague Dawley albino rats (n = 25; 6–8 weeks old) were provided by the Department of Pharmacology, Animal House, University of Port Harcourt, Rivers State, Nigeria. All rats were kept in polypropylene cages under usual room temperature of 25 ± 2 °C with a 12-h light/dark cycles throughout the entire duration of the experiment. Before the beginning of this study, the animals were acclimatized for fourteen days. The experimental animals (n = 25) were divided into five groups and each group consisted of five animals. Controls (group 1) were orally treated with distilled water for 60 days; next, group 2 was exposed to heavy metal mixture (HMM) with following concentrations (20 $mg{\cdot}kg^{-1}$ of Pb body weight (Institóris et al., 2006a); 0.40 $mg{\cdot}kg^{-1}$ of Hg (Institóris et al., 2006a); 0.56 $mg{\cdot}kg^{-1}$ of Mn; and 35 $mg{\cdot}kg^{-1}$; of Al (Su et al., 2017). while groups 3,4 and 5 were exposed to HMM and co-treated with zinc chloride ($ZnCl_2$; 0.80 mg/kg)(Anyanwu et al., 2020a), sodium selenite (Na2SeO3;1.50 mg/kg)(Messarah et al., 2012) and both (zinc chloride and sodium selenite) respectively. The heavy-metal compounds were separately dissolved as a stock solution before use to avoid precipitation and diluted to the working concentration with ultrapure water. Environmentally relevant doses of metal mixtures deduced from recently conducted studies from various matrices in the Niger Delta, Nigeria were used in this study (Okoye et al. 2022). Exposure doses of metals mixture in this study were consider as low (Institóris et al., 2006b), moreover, they are at lower ranges when compared to doses of metals used in a similar in vivo studies (Andjelkovic and Buha Djordjevic, 2019, Zhou et al., 2020a).

Rats were weighed weekly and daily feed and fluid intakes were recorded. After 60 days of exposure, animals were euthanized by intraperitoneal administration of the pentobarbital (50 mg/kg). The cerebral and cerebellum of every rat were dissected, weighed, and used for both biochemical parameters and heavy metal analyses.

The ethical approval was obtained from the University of Port Harcourt institutional Centre for Research Management and Development Animal Care and Use Research Ethics Committee (UPH/PUTOR/REC/12). The experiment was conducted in accordance with the "Guide for the Care of Laboratory Animals" approved by the National Academy of Science (NAS). The animals received standard feed and deionized water ad libitum.

Passive Avoidance Test

Passive avoidance test was performed using a shuttle box after the sixtieth day of experiment. The apparatus consisted of light and dark compartments, interconnected by a guillotine door. Each rat was placed separately in the light compartment for one minute (sixty seconds) during the training process. When the door was opened, the rat moved into the dark compartment, thereafter the door was closed, and the animal received foot electric shock (0.5 mA) for 3 s via the stainless grid floor. After the training is done, test session began; during the testing, each rat was again placed into the light compartment. Animals were observed as they responded by returning to the light compartment after being shocked by an adverse stimulus. The time-through latency to entering the dark compartment was measured as a positive index of memory performance, with a 300 s cut-off time. We utilized this protocol for the Passive Avoidance Test in our previous research (Ikpeama et al., 2023).

Sample collection and brain tissue preparation

The rats were euthanized using pentobarbital (intraperitoneal administration; dose of 50 mg/kg), and the brain samples were quickly dissected and afterwards removed from the rest of the central nervous system; Two telencephalic hemispheres (left and right) and cerebellum were stored at − 80 °C for metal and biochemical analysis.

The cerebral cortex and cerebellum were separately homogenized in 9 vol of cold phosphate buffer (0.1 M: pH 7.4) using homogenizer. The tissue homogenates were centrifuged at 3000 rpm for 20 min at 4 °C to separate the nuclear debris. The cerebral cortex and cerebellum lysates were used for the assay of Acetylcholinesterase (AChE), MDA, NO, GSH, GPx, GST, SOD and for ELISA assays [(Tumour necrosis factor alpha

('TNF-α), Interleukin-6 (IL-6), Nuclear factor E2-related factor 2 (Nrf2), Factor Kappa B (NF-kB), Hmox-1, Casp-3).

ELISA analysis

The assay that determined the levels of cytokines, transcription factors, apoptotic marker in the cerebral cortex and cerebellum were previously described in another study (Eddie-Amadi et al., 2022). The following pro-inflammatory cytokines (IL-6 and 'TNF-α), transcription factor (NF-kB and Nrf2), (Hmox1), and apoptotic biomarker (Caspase 3) were analysed by the instructions provided by the manufacturer's (Elab science Biotechnology Company (Beijing, China). ELISA kit (Elabscience science Biotechnology Company,(Beijing, China) was used to measure the pro-inflammatory cytokines (IL-6 and 'TNF-α), transcription factor (NFKB-p105 and Nrf2), (Hmox1), and apoptotic biomarker (Caspase 3) concentrations of supernatants according to the manufacturer's instructions.

Antioxidant and Oxidative stress markers

Glutathione related antioxidants (GPx), Reduced glutathione (GSH), Glutathione S-transferase (GST), Superoxide Dismutase (SOD), Catalase, Lipid peroxidation, Nitric oxide

GPx was measured using well-established method of Paglia and Valentine (Paglia and Valentine, 1967). The absorbance was measured at 412 nm; GSH was analysed by the method of Jollow et al. (Jollow et al., 1974) and absorbance was measured at 412 nm as well. GST activity was evaluated by the method of Habig et al.(Jollow et al., 1974). The absorbance (At) was measured against air at 310 nm using the following equation: GST activity (μmol min^{-1} mg^{-1} protein) = At / (1.9 × time x mg protein). SOD activity was assayed using 20 μl of the cerebral cortex and cerebellum (separately) supernatant sample (test) or buffer (reference) and 10 μl pyrogallol (20 mM in 10 mM HCl) to which, 1 ml buffer solution was added (Marklund and Marklund, 1974). The absorbance of test (At) or reference (Ar) was measured at 420 nm against air after 30 s and 90 s. The percentage inhibition of pyrogallol autoxidation was calculated according to the following equation: The percentage inhibition = [100 – (At $min^{-1}ml^{-1}$ sample)/(Ar $min^{-1}ml^{-1}$ reference)] x 100. Catalase activity was estimated by monitoring the rate of H_2O_2 breakdown at 240 nm according to Aebi's method (Bergmeyer et al., 1974). Briefly, 990 μl of catalase buffer (0.036% H_2O_2 prepared in 50 mM phosphate buffer, pH 7.0) was added to 10 μl of cerebral cortex and cerebellum lysates separately in a cuvette. Catalase activity was assayed immediately at 240 nm for 3 min and expressed as μmol/min/mg protein.

Lipid peroxidation was analysed as thiobarbituric acid reactive substances (TBARS) by the adaptation of Esterbauer and Cheeseman method (Esterbauer and Cheeseman, 1990). In a nutshell, 500 μl of CC and CE separately supernatant was added to one ml TCA (20%) and mixed thoroughly. The mixture was centrifuged at 3000 rpm for 10 min. One ml of the supernatant was added to 0.5 ml of 0.7% TBA and allowed to boil for 10 min. After cooling, the absorbance was read at 532 nm against blank.

Nitric oxide (NO): this assay adapted the Griess reaction technique (Sosroseno et al., 2008, Oktem et al., 2012). One microliter 100 μl of CC and CE (separately) supernatant was added to 100 μl acidic Griess reagent (1% sulfanilamide and 0.1% naphthlethylenediamine dihydrochloride in 2.5% phosphoric acid). The absorbance was read at 540 nm against blank.

Determination of heavy metals and essential minerals

Harvested brain structures were dried for 48 h, and were weighed and placed in 10 ml conical flasks with polypropylene lids containing 3 ml of HNO_3 at room temperature until the solution became clear. Then, 1 ml of 30% H_2O_2 was added to the samples. At the end of effervescence, the samples were heated at 80 °C to remove the HNO3, cooled to room temperature and final volume was made up to 10 ml with 2% HNO_3. The samples were brought to a constant volume.

Cerebral cortex and cerebellum Al, Mn, Pb and Hg were determined with an Atomic Absorption Spectrometer (Okoye et al., 2022).

Histopathological examination

After the behavioral test, the animals were sacrificed and transcardiac perfusion with heparinized saline 0.9% solution was done followed by 4% paraformaldehyde in 0.2 M phosphate buffer. in paraffin. The cerebral cortex and cerebellum from all the experimental groups were fixed in 10% formaldehyde, dehydrated in graded alcohol, cleared in xylene and then embedded in paraffin.

The embedded,brain parts were sectioned into 5 μm slices for Toluidine blue staining. The histological examination is blindly evaluated by two experienced histopathologists.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Microsoft Xlstat 2014 was used in performing Analysis of Variance and Tukey multiple comparison pairwise tests to check if the concentration of the biomarkers was significantly different between groups. Pandas was used in obtaining the descriptive statistical parameters (biomarkers and metals mean concentrations) for the various rat organs. Seaborn and Matplotlib were used in plotting all graphs. The data analysis involved performing descriptive statistics of the metals and biomarkers concentration before ANOVA was used to establish if there was significant difference in the concentration of the heavy metals and biomarkers among groups. All significant differences were at a $p < 0.05$. Pearson R correlation was used to understand the relationship among biomarkers.

Results

Effect of essential metals on the body weight, absolute and relative weight of cerebellum (C) and cerebral cortex (CC) of female albino rats exposed to HMM

At the end of the experiment, controls had higher body weight than HMM exposed rats ($p < 0.05$) and rats co-treated with HMM and Zn, Se and Zn+Se ($p < 0.05$). HMM + Se and HMM + Se + Zn groups had similar body weight when compared to each other ($p > 0.05$); however, both groups had higher body mass compared to HMM + Se or HMM + Se + Zn groups ($p < 0.05$).

We obtained that absolute weight of cerebellum did not vary between HMM exposed animas and controls, nor between HMM exposed animas and rats co-treated with HMM and Zn, Se and Zn +Se ($p > 0.05$). On other side, Absolute weight of cerebral cortex was higher in rats co-treated with HMM and Zn, Se and Zn +Se compared to HMM only exposed ($p < 0.05$) or controls ($p < 0.05$). Nevertheless, absolute weight of cerebral cortex was similar between HMM treated rats and controls ($p > 0.05$).

Food and fluid intake did not vary between examined groups at the end of experiment ($p > 0.05$) (Table 1).

Effects of Zn and Se on Al, Pb, Mn and Hg bioaccumulation in cerebellum (C)

The result of the effect of essential trace elements on lead accumulation in the cerebellum (C) is presented in Fig. 1**A**. The rats exposed to HMM + Zn had a mean value of 0.872 ± 0.018 mg/kg, then rats exposed to HMM + Se had a mean value of 0.918 ± 0.004 mg/kg, while rats exposed to HMM + Zn + Se had a mean value of 0.846 ± 0.012 mg/kg. Turkey multiple comparisons showed that the lead accumulated in

Table 1
Effect of essential metals on the body weight, absolute and relative weight of cerebellum (C) and cerebral cortex (CC) of female albino rats exposed to HMM; (n = 25, n = 5/group).

Group	Absolute Organ Weight (g) (C)	Relative Weight (%) (C)	Absolute Organ Weight (g) (CC)	Relative Weight (%) (CC)	Brain Weight (g)	Body Weight (g)	Feed intake	Fluid intake
Control	0.30±0.00[a]	18.75	0.57±0.08[a]	35.63	1.60±0.42	200.00±14.14[a]	154.10±23.42[a]	242.98±35.36[a]
Heavy Metal Mixture	0.25±0.07[a]	13.89	0.60±0.00[a]	33.33	1.80±0.00	180.50±0.71[b]	148.27±16.42[a]	210.07±33.46[a]
HMM + Zn 0.8 mg/kg	0.24±0.02[a]	14.55	0.80±0.02[b]	48.48	1.65±0.00	162.50±12.02[b]	147.94±22.13[a]	195.62±43.20[a]
HMM + Se 1.5 mg/kg	0.20±0.07[a]	12.34	0.81±0.02[b]	50.00	1.62±0.15	155.50±0.71[c]	127.63±35.47[a]	209.74±51.27[a]
HMM + Zn + Se	0.23±0.01[a]	14.38	0.86±0.06[b]	53.75	1.60±0.02	155.00±7.07[c]	127.82±29.85[a]	203.85±53.26[a]

Values are presented as Mean ± SD values with different superscripts are significantly different from each other at $p < 0.05$), while values with the same superscripts are not significantly different.

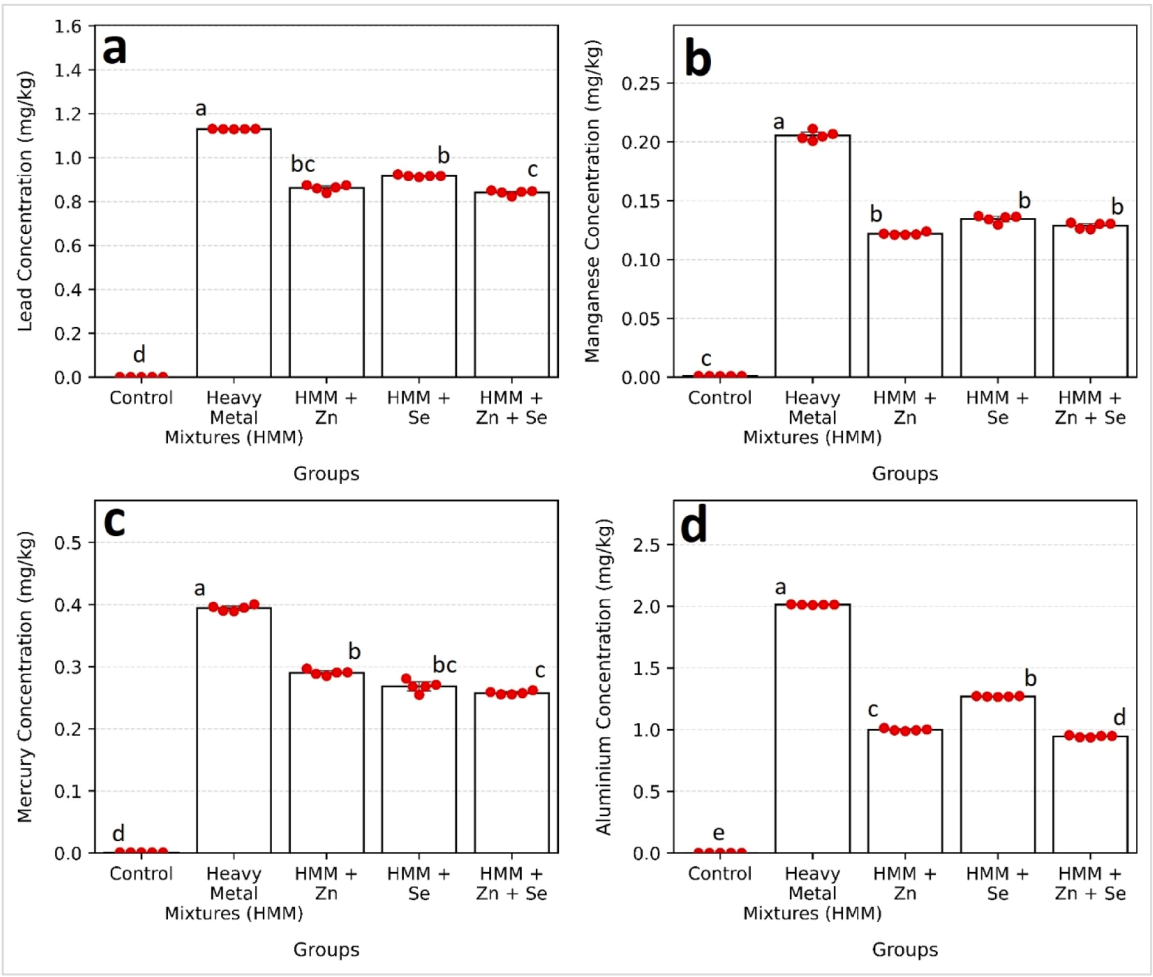

Fig. 1. Effect of essential metals on the bioaccumulation of A) lead, B) manganese C) mercury and D) aluminum in the cerebellum. Different superscripts indicate a statistically significant difference between the means at P-values < 0.05." This means that if two means have different superscripts next to them (e.g., "a" and "b"), it indicates that there is a statistically significant difference between the two means at a significance level of 0.05 or less. While same superscripts have no significant difference. This means that if two means have the same superscript next to them (e.g., "a" and "a"), it indicates that there is no statistically significant difference between the two means at a significance level of 0.05 or less.

the essential metal treatment groups were significantly lower compared to HMM rats ($p < 0.05$). Essential trace elements treatment groups compared with group 2 (HMM treated only) showed a least percentage reduction of 18.84 in the rats administered with HMM + Se, the highest percentage reduction of lead was 25.21 in rats exposed to HMM + Zn + Se, while rats exposed to HMM + Zn had a percentage reduction of 22.91. The ANOVA revealed statistically significant difference in the levels of manganese among the groups ($p < 0.05$); moreover, HMM rats had significantly higher levels of manganese in CE compared to HMM + Zn, HMM + Se, HMM + Zn + Se groups and controls ($p < 0.05$). Interestingly, HMM + Zn, HMM + Se groups did not vary in Mn levels when compared to each other ($p > 0.05$). Also, controls had significantly lower levels of Mn in cerebellum compared to groups treated with essential elements (groups 3,4, and 5). We obtained following

percentage reduction in Mn levels 38.94, 34.86 and 41.11 for groups HMM + Zn, HMM + Se and HMM + Se + Zn respectively compared to HMM exposed rats (group 2).

The ANOVA revealed statistically significant difference in the levels of mercury among the groups ($p < 0.05$). HMM + Zn and HMM + Se groups had similar levels of Hg in cerebellum ($p > 0.05$) when compared to each other, lower Hg levels than HMM treated group (group 2) ($p < 0.05$) and higher Hg levels than HMM+ Zn+ Se and controls ($p < 0.05$). Rats treated with HMM + Zn as had a Hg concentration of 0.290 ± 0.003 mg/kg in CE, then rats exposed to HMM + Se had a mercury value of 0.270 ± 0.004 mg/kg in CE, while rats exposed to HMM + Zn + Se had a mean Hg value of 0.257 ± 0.004 mg/kg. HMM + Zn rats showed a percentage reduction of 27.53 while HMM + Se rats showed a percentage reduction of 32.54 and HMM + Se + Zn rats showed a percentage reduction of 35.67 compared to HMM treated rats (group 2).

The rats exposed to HMM and Zinc had a mean Al level in CE 0.997 ± 0.007 mg/kg; then rats exposed to HMM and selenium had a mean Al value of 1.269 ± 0.002 mg/kg in CE, while finally, rats exposed to HMM and co-treated with Zinc and Selenium had 0.940 ± 0.007 mg/kg of Al in CE. Turkey multiple comparisons showed that the aluminum accumulated in the essential metal treatment groups were significantly different from HMM rats ($p > 0.05$). Essential metal treatment groups compared with group 2 revealed a least percentage reduction of 37.01 in the rats administered with HMM + Se, the highest percentage reduction of lead was 53.36 in rats exposed to HMM + Zn + Se, while rats exposed to HMM + Zn had a percentage reduction of 50.51. HMM treated group (group 2) had significantly higher concentrations of Pb, Hg, Al and Mn in cerebellum compared to groups 3,4 and 5 (essential elements treated groups); nevertheless, HMM + Zn, HMM + Se and HMM + Zn + Se had higher levels of Pb in the cerebellum compared to controls ($p < 0.05$).

Effects of Zn and Se on Al, Pb, Mn and Hg bioaccumulation in the cerebral cortex

The effect of the treatment of accumulated lead with essential metals in cerebral cortex is presented in Fig. 2. Turkey multiple comparison showed that the lead accumulated in the essential metal treatment groups were significantly different from HMM rats ($p < 0.05$). HMM + Zn exposed rats had a mean concentration of Pb 0.987 ± 0.005 mg/kg in CC, HMM + Se rats had 1.098 ± 0.004 mg/kg of Pb in CC while HMM + Zn + Se rats had 0.912 ± 0.011 mg/kg of Pb in CC.

HMM + Zn treated rats had a mean Mn value of 0.316 ± 0.004 mg/kg in CC, then HMM + Se rats had 0.324 ± 0.002 mg/kg of Mn in CC while HMM + Zn + Se rats had 0.303 ± 0.002 mg/kg of Mn in CC. When compared with HMM only exposed rats, the essential metals groups (groups 3,4 and 5) had a percentage reduction of 27.94 in HMM + Zn rats, 26.23 in the HMM + Se and 31.01 in the HMM + Zn + Se. Turkey multiple comparison test for manganese revealed significant difference among the examined groups ($p < 0.05$). HMM + Zn treated rats had 0.404 ± 0.002 mg/kg of Mn in CC, then HMM + Se exposed rats had 0.393 ± 0.006 mg/kg of Mn in CC, while HMM + Zn + Se treated rats had 0.318 ± 0.006 mg/kg of Mn in CC. The ANOVA testing showed significant accumulation of mercury in the Cerebral cortex (CC)

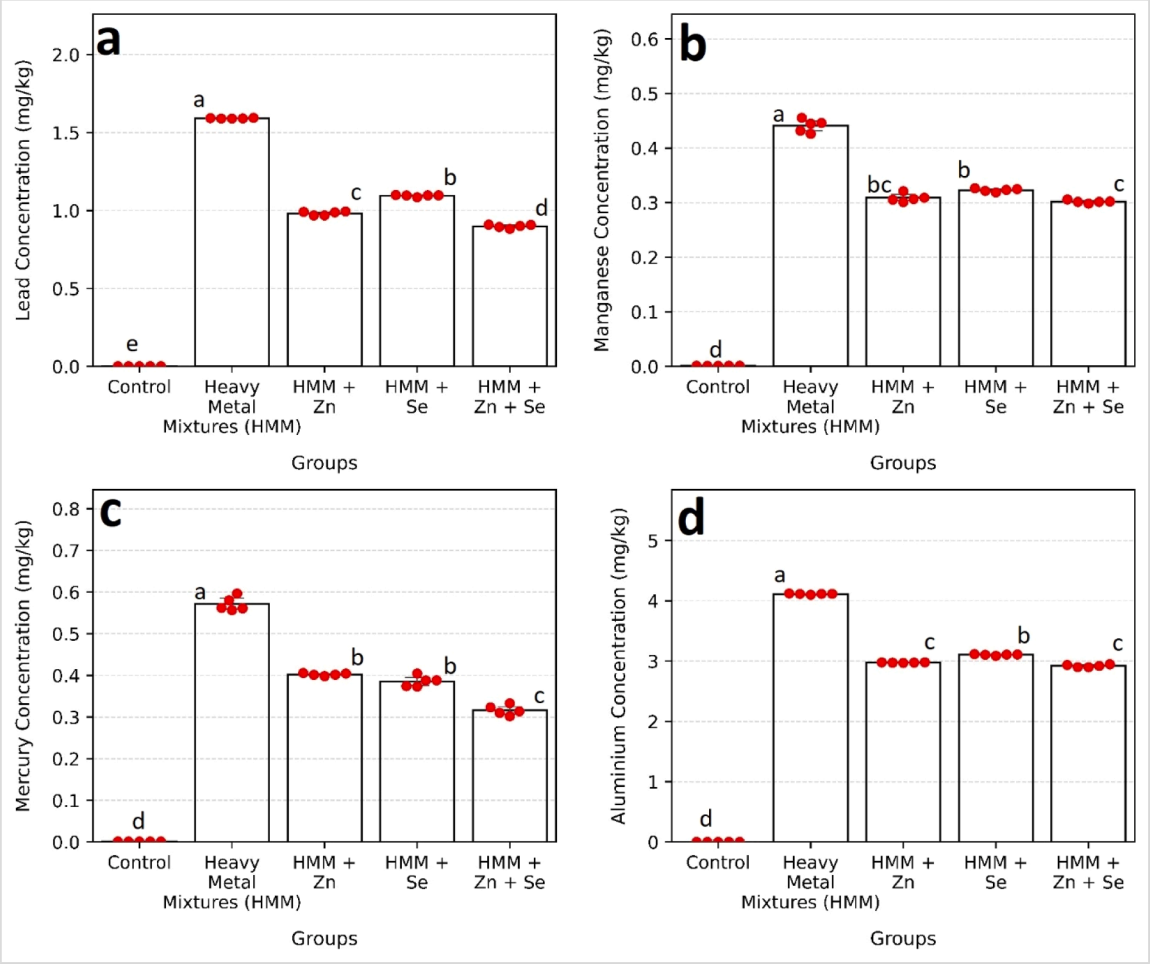

Fig. 2. Effect of essential metals on the bioaccumulation of A) lead, B) manganese C) mercury and D) aluminum in the cerebral cortex. Values are expressed as Mean ± SD. Different superscripts indicate a statistically significant difference between the means at P-values < 0.05 while same superscript have no significant difference.

of rats that were significantly different among the groups ($p < 0.05$). HMM + Zn exposed rats had 2.979 ± 0.002 mg/kg of Hg in CC, then HMM + Se treated rats had 3.111 ± 0.007 mg/kg of Hg in CC while HMM + Zn + Se exposed animals had 2.954 ± 0.021 mg/kg of Hg in CC.

HMM treated group (group 2) had significantly higher concentrations of Pb, Hg, Al and Mn in cerebral cortex compared to groups 3,4 and 5 (essential elements treated groups); nevertheless, HMM + Zn, HMM + Se and HMM + Zn + Se had higher levels of Pb in the CC compared to controls ($p < 0.05$).

Effects Heavy metal mixtures (HMM) (Pb, Mn, Hg and Al) and Essential element (Zn and Se) exposure on antioxidants (SOD, GPx, CAT, GSH) and MDA (μmol/ml) and NO (μM/l) levels in cerebellum (C) and cerebral cortex (CC) of female rats

We found lower activity of SOD in the HMM, HMM + Zn, HMM + Se and HMM + Se + Zn groups compared to controls in C ($p < 0.05$); nevertheless, levels of SOD in the HMM + Zn were higher than in HMM, HMM + Se and HMM + Se + Zn groups ($p < 0.05$). Levels of SOD in the cerebral cortex and GPx in the cerebellum did vary between the examined groups ($p > 0.05$). In contrast, in the CC, HMM treated animals had lower levels of GPx (<0.05), while, groups treated with essential metals had higher levels of GPx compared to HMM exposed rats ($p < 0.05$) but lower GPx levels than controls ($p < 0.05$). We obtained that levels of CAT were similar between controls and HMM + Se + Zn rats ($p > 0.05$) in cerebellum, while controls had higher activity of CAT in than HMM + Se and HMM + Zn and HMM groups ($p < 0.05$); also groups treated with HMM and Zn had higher levels of CAT than HMM and HMM + Se animals ($p < 0.05$). In cerebral cortex, controls had higher CAT levels compared to HMM + Se + Zn rats ($p < 0.05$), while both of them (controls and HMM + Zn + Se) had higher levels of CAT than HMM, HMM + Zn and HMM + Se animals ($p < 0.05$). As for the cerebellum, we found that HMM treated rats as well as all three groups of animals exposed to the essential metals had lower activity of GSH compared to the controls ($p < 0.05$). Moreover, HMM + Se, HMM + Zn and HMM + Se + Zn had higher levels of GSH than HMM exposed rats ($p < 0.05$), while in the cerebral cortex, GSH levels did not vary between HMM, HMM + Se, HMM + Zn and HMM + Se + Zn ($p > 0.05$) but all four treated groups had lower GSH activity compared to controls ($p < 0.05$).

As for the cerebellum and cerebral cortex, we obtained that levels of MDA were significantly higher in HMM group than in controls ($p < 0.05$), while HMM + Zn+ Se, HMM + Zn and HMM + Se groups had lower MDA levels than HMM exposed rats ($p < 0.05$) but higher than controls ($p < 0.05$). We found that levels of NO were significantly higher in HMM group than in controls ($p < 0.05$), while HMM + Zn+ Se, HMM + Zn and HMM + Se groups had lower NO levels than HMM exposed rats ($p < 0.05$) but higher than controls ($p < 0.05$) but only in the cerebellum. As for the cerebral cortex, NO levels were also higher in HMM and HMM + Se treated rats compared to controls ($p < 0.05$), while HMM + Zn and HMM + Zn + Se rats had lower levels of NO than HMM and HMM + Se treated rats. Table 2.

Effect of essential metals (Zn and Se) on the pro-inflammatory cytokines (IL-6, (pg/ml)), (TNF-α, (pg/ml)), Transcription factors (Nrf2, (pg/ml)), (Nfkb, (pg/ml)), and Caspase 3 (μmol/ml) in cerebellum (C) and cerebral cortex (CC) of female albino rats after heavy metal mixtures (Pb, Mn, Hg and Al) exposure

We obtained that IL-6 and TNF- α were higher in HMM group compared to controls ($p < 0.05$), while groups co-treated with HMM and Zn, Se and Zn + Se had lower level of both, IL-6 and TNF- α in the cerebral cortex and cerebellum (Table 3). Next, we found that HMM group had significantly lower expression of Nrf2 compared to controls in the cerebral cortex and cerebellum ($p < 0.05$). All three co-treated groups (HMM + Zn, HMM + Se and HMM + Zn + Se) had higher levels of Nrf2 than HMM exposed animals ($p < 0.05$) in the cerebellum, while in the cerebral cortex, only HMM + Zn + Se group had higher levels of Nrf2 than controls ($p < 0.05$), while other two groups (HMM + Zn and HMM + Se) did vary when compared to HMM treated rats ($p > 0.05$). Expression of NF+kB was lower in HMM exposed animals compared to controls ($p < 0.05$) in the cerebellum and cerebral cortex; however, in both examined CNS areas, only HMM + Se and HMM + Zn + Se rats recovered levels of the NF-kB, e.i. had higher levels of NF-kB than HMM treated rats ($p < 0.05$), while HMM + Zn and HMM groups had similar levels of it ($p > 0.05$). Casp3 levels were higher in HMM treated animals compared to all other four groups in both examined areas ($p < 0.05$).

Effect of Essential element on the AChE (μmol/ml) activity in Cerebellum and Cerebral cortex of female albino rats after heavy metal mixtures (Pb, Mn, Hg, and Al) exposure

We found increased acetylcholinesterase levels in HMM treated rats compared to controls ($p < 0.05$) in the cerebral cortex and cerebellum. Nevertheless, HMM + Zn and HMM + Zn + Se groups had lower AChE levels than the HMM group in both examined areas (cerebellum and cerebral cortex) ($p < 0.05$). Table 4.

Effect of essential metals on Passive avoidance test

Rats exposed to HMM had significantly less escape dormancy compared to rats in the control group ($p < 0.05$) or compared to HMM and essential metals co-treated rats ($p < 0.05$); moreover, rats co-treated

Table 2
Effects Heavy metal mixtures (HMM) (Pb, Mn, Hg and Al) and Essential element (Zn and Se) exposure on antioxidants (SOD, GPx, CAT, GSH) and MDA (μmol/ml) and NO (μM/l) levels in cerebellum (C) and cerebral cortex (CC) of female albino rats (n = 25).

Treatment	SOD		GPx		CAT		GSH		MDA		NO	
	C	CC	C	CC	C	CC	C	CC	C	CC	C	CC
Control	0.37 ± 0.08^{b}	0.25 ± 0.13^{a}	0.08 ± 0.00^{a}	0.07 ± 0.01^{a}	1.39 ± 0.37^{b}	0.90 ± 0.11^{a}	2.18 ± 0.06 ^{n}	1.70 ± 0.10^{a}	0.34 ± 0.04^{c}	0.37 ± 0.01^{b}	1.19 ± 0.73^{b}	1.95 ± 0.07^{b}
HMM	0.16 ± 0.01^{a}	0.19 ± 0.02^{a}	0.04 ± 0.00^{a}	0.02 ± 0.01^{b}	0.62 0.01^{a}	0.45 ± 0.05^{b}	0.56 ± 0.16^{a}	0.58 ± 0.03^{b}	0.91 ± 0.01^{a}	0.78 ± 0.11^{a}	3.95 ± 0.63^{a}	3.42 ± 1.77^{a}
HMM + Zn 0.8 mg/kg	0.21 ± 0.08^{ab}	0.21 ± 0.11^{a}	0.07 ± 0.02^{a}	0.04 ± 0.00^{ab}	1.02 ± 0.41^{ab}	0.58 ± 0.06^{b}	1.45 ± 0.52^{b}	0.66 ± 0.10^{b}	0.55 ± 0.04^{bc}	0.60 ± 0.11^{ab}	1.74 ± 0.88^{ab}	2.71 ± 0.45^{ab}
HMM + Se 1.5 mg/kg	0.17 ± 0.00^{a}	0.15 ± 0.00^{a}	0.06 ± 0.01^{a}	0.04 ± 0.00^{ab}	0.87 ± 0.30^{a}	0.60 ± 0.06^{b}	1.32 ± 0.58^{b}	0.68 ± 0.23^{b}	0.58 ± 0.01^{bc}	0.61 ± 0.01^{ab}	1.87 ± 0.42^{ab}	3.40 ± 0.14^{a}
HMM + Zn + Se	0.17 ± 0.06^{a}	0.18 ± 0.02^{a}	0.06 ± 0.02^{a}	0.05 ± 0.01^{ab}	1.10 ± 0.30^{b}	0.67 ± 0.00^{ab}	1.38 ± 0.66^{b}	0.77 ± 0.01^{b}	0.59 ± 0.12^{b}	0.57 ± 0.04^{ab}	1.96 ± 0.65^{ab}	2.34 ± 1.04^{b}

Values are presented as Mean ± standard deviation values with different superscripts are significantly different from each other at $p < 0.05$, while values with the same superscripts are not significantly different (n = 25, n = 5/group).

Table 3
Effect of essential metals (Zn & Se) on the Pro-inflammatory cytokines (IL-6, (pg/ml)), (TNF-α, (pg/ml)), Transcription factors (Nrf2, (pg/ml)), (Nfkb, (pg/ml)), and Caspase 3 (μmol/ml) in cerebellum (C) and cerebral cortex (CC) of female albino rats after heavy metal mixtures (Pb, Mn, Hg and Al) exposure. Values are presented as Mean ± SD values Different superscripts indicate a statistically significant difference between the means at P-values < 0.05." This means that if two means have different superscripts next to them (e.g., "a" and "b"), it indicates that there is a statistically significant difference between the two means at a significance level of 0.05 or less. While same superscripts have no significant difference. This means that if two means have the same superscript next to them (e.g., "a" and "a"), it indicates that there is no statistically significant difference between the two means at a significance level of 0.05 or less; n = 25.

Treatment	IL-6		TNF-α		Nrf2		NF-kB		Casp3	
	C	CC	C	CC	C	CC	C	CC	C	CC
Control	4.15 ± 2.33^{a}	7.20 ± 1.27^{a}	26.20 ± 14.99^{a}	13.00 ± 0.71^{a}	31.50 ± 3.39^{a}	19.65 ± 0.78^{a}	0.14 ± 0.02^{a}	0.09 ± 0.00^{a}	0.26 ± 0.13^{a}	0.12 ± 0.02^{b}
HMM	15.65 ± 6.29^{b}	11.95 ± 2.47^{b}	60.90 ± 16.83^{b}	45.25 ± 27.22^{b}	4.25 ± 0.07^{c}	7.90 ± 1.13^{b}	0.05 ± 0.00^{b}	0.05 ± 0.00^{b}	0.65 ± 0.03^{b}	0.63 ± 0.03^{a}
HMM + Zn 0.8 mg/kg	5.95 ± 3.49^{a}	6.45 ± 1.77^{a}	36.35 ± 2.90^{a}	21.05 ± 9.26^{a}	10.95 ± 1.48^{bc}	9.05 ± 1.34^{b}	0.10 ± 0.02^{b}	0.06 ± 0.00^{b}	0.44 ± 0.03^{a}	0.15 ± 0.07^{b}
HMM + Se 1.5 mg/kg	4.95 ± 4.17^{a}	7.60 ± 3.54^{a}	35.90 ± 11.03^{a}	22.95 ± 3.75^{a}	10.15 ± 1.63^{bc}	10.00 ± 0.57^{b}	0.08 ± 0.02^{ab}	0.07 ± 0.01^{ab}	0.47 ± 0.23^{a}	0.15 ± 0.06^{b}
HMM + Zn + Se	11.85 ± 4.31^{a}	8.70 ± 1.13^{a}	37.15 ± 33.45^{a}	20.70 ± 1.13^{a}	20.70 ± 4.81^{ab}	16.10 ± 1.56^{a}	0.11 ± 0.00^{a}	0.09 ± 0.00^{a}	0.41 ± 0.08^{a}	0.13 ± 0.04^{b}

Table 4
Effect of Essential element on the AChE (μmol/ml) activity in the Cerebellum and Cerebral cortex of female albino rats after heavy metal mixtures (Pb, Mn, Hg, and Al) exposure; (n = 25, n = 5/group).

Treatment	AChE	
	C	CC
Control	110 ± 28.28^{b}	110 ± 28.28^{b}
Heavy Metal Mixture (HMM)	230 ± 14.14^{a}	210 ± 14.14^{a}
HMM + Zn 0.8 mg/kg	165 ± 35.36^{b}	130 ± 14.14^{b}
HMM + Se 1.5 mg/kg	220 ± 56.57^{a}	160 ± 42.43^{a}
HMM + Zn + Se	220 ± 42.43^{a}	135.5 ± 36.06^{ab}

with essential trace element after being exposed to toxic metal mixture showed significantly ($p < 0.05$) lower escape dormancy than controls ($p < 0.05$). The groups that were concurrently treated with essential trace element did not significantly differ from one another ($p > 0.05$) (Fig. 3).

Effect of Essential element on the histology of Cerebral cortex (CC) of female albino rats after heavy metal mixtures (Pb, Cd, Hg and As) exposure

Fig. 4a, Group 1 that received deionized water only showed cerebral cortex with normal pyramidal cells (PMC). Fig. 4b, Group 2 received HMM only had cerebral cortex showing neuronal cells loss and vacuolation. Fig. 4c, Group 3 is photomicrograph section of cerebral cortex section from rat treated with HMM plus zinc. The section showed recovered and organized pyramidal cells. Fig. 4d, Group 4 (HMM plus Se): Section showed organized cortical layers (OCLN) and recovered pyramidal cells. Fig. 4e Group 5: Photomicrograph of a section of cerebral cortex from rat brain treated with HMM plus selenium plus zinc. Section showed regenerated and reorganized and neuronal cells (RRN).

Fig. 5a Group 1 (Control deionized) Section showed the molecular Layer with basket cells (BC), purkinje Layer (PL) and purkinje cells (PC) and granular layer with round cells (RC). Fig. 5b, Group 2, (HMM only). There was vacuolation in the Purkinje layer with disorganized cell layers

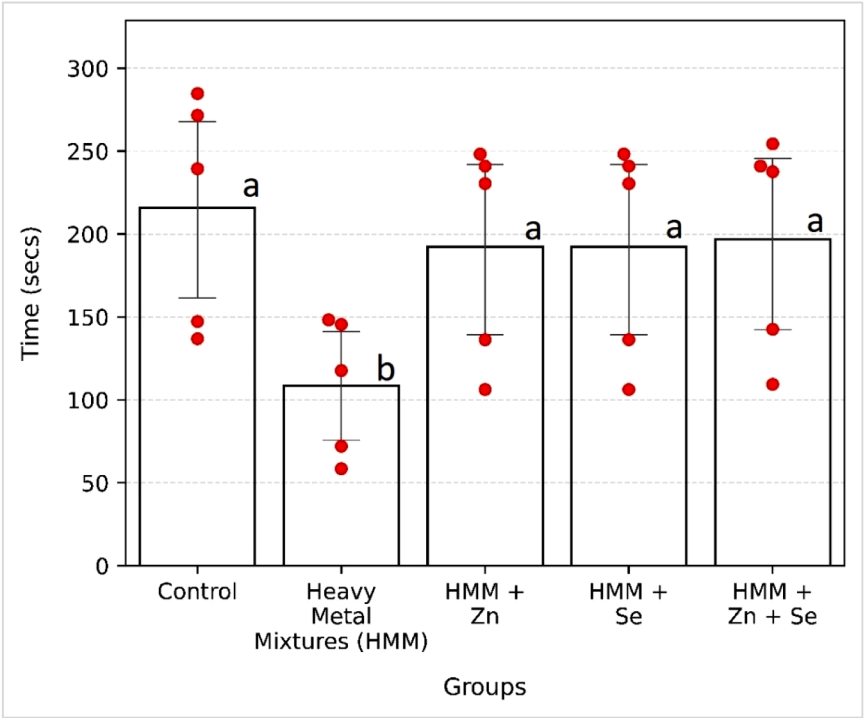

Fig. 3. Effect of essential metals on Passive avoidance test; Different superscripts indicate a statistically significant difference between the means at P-values < 0.05." This means that if two means have different superscripts next to them (e.g., "a" and "b"), it indicates that there is a statistically significant difference between the two means at a significance level of 0.05 or less. While same superscripts have no significant difference. This means that if two means have the same superscript next to them (e.g., "a" and "a"), it indicates that there is no statistically significant difference between the two means at a significance level of 0.05 or less.

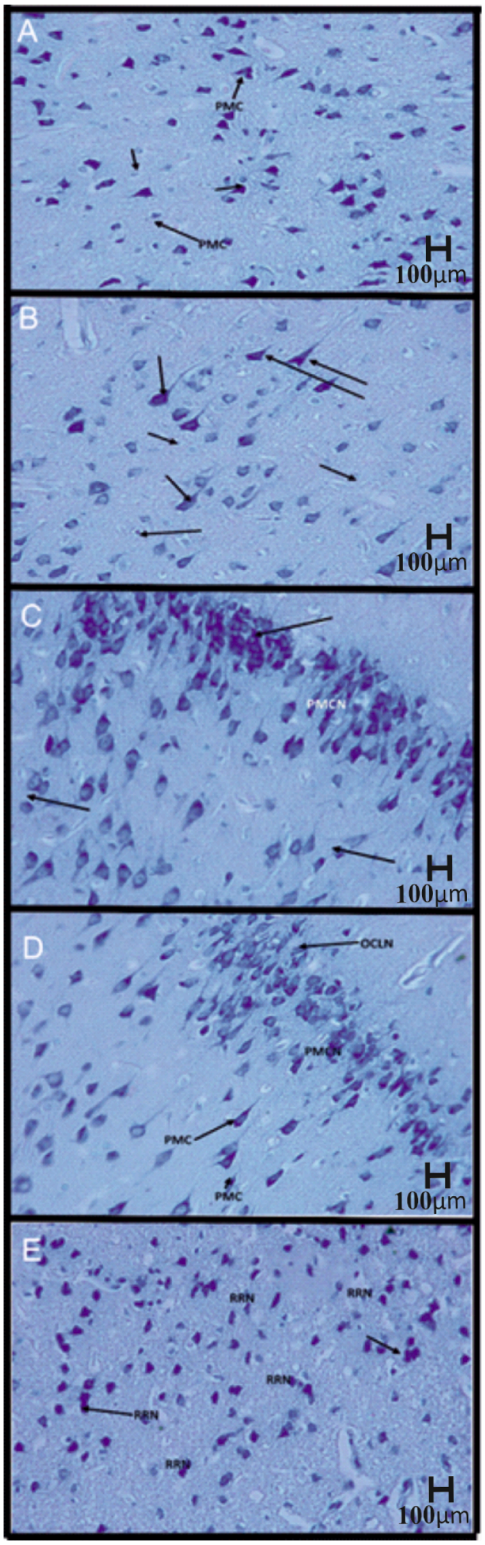

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Fig. 4. The cerebral cortex was studied under different conditions, as illustrated in Fig. 4A. In Group 1 (control, deionized water), a photomicrograph section of the cerebral cortex showed normal pyramidal cells (PMC) at a magnification of 400x. In Group 2 (HMM only; B), a photomicrograph section of the cerebral cortex showed neuronal cell loss and vacuolation at 400x magnification. In Group 3 (HMM plus Zinc; C), a photomicrograph section of the cerebral cortex from a rat treated with heavy metal plus zinc was examined. The section showed recovered and organized pyramidal cells at 400x magnification. Group 4 (HMM plus Se; Fig. 4D) displayed a photomicrograph section of rat tissues from the brain treated with heavy metal and selenium. The section showed organized cortical layers (OCLN) and recovered pyramidal cells at a magnification of 400x. Lastly, Group 5 (HMM plus Zn plus Se, Fig. 4E) demonstrated a photomicrograph section of the cerebral cortex from a rat treated with HMM plus selenium plus zinc. The section showed regenerated and reorganized neuronal cells (RRN) at 400x magnification.

and neuronal darkening). Fig. 5c Group 3 (HMM plus Zinc) showing organized molecular layer, Purkinje layer and Granular Layer (GC) and regenerations of the cells. Fig. 5d Group 4e, (HMM plus Se). The photomicrograph cerebellum of rats treated with HMM plus selenium showed organized cytoarchitecture of the molecular layer (ml), degenerated Purkinje layer DPL and granular layer (GL). Fig. 5e (HMM plus Se plus Zn) Photomicrograph section of brain tissue from cerebellum of rat treated with heavy metal plus zinc and selenium showed reorganized and regenerated 3 layers of the cerebellar.

Discussion

This study has shown that exposure to HMM- induced reduction in the levels of antioxidants in the cerebral cortex and cerebellum, while co-treatment with Se and Zn significantly improved activity of antioxidants but still not as it was in the controls. In our study, mixture of Al, Pb, Mn and Hg generated lipid peroxidation in the cerebellum and cerebral cortex, and this was manifested as increased levels of MDA and NO; nonetheless, this adverse effects of HMM were mitigated by the presence of Zn and Se. Our results indicated that exposure to HMM induced drop of cellular levels of Nrf2, transcription factor that controls the expression of a large pool of antioxidant such as GPx (Banning et al., 2005) or SOD (Zhang et al., 2012), antioxidant enzymes which we also found to be downregulated in the presence of HMM.

Heavy Metals bioaccumulation

All four metals that were used in this study accumulated in the cerebellum and cerebral cortex (Al, Pb, Hg and Mn) in higher extent, compared to controls; similar findings were obtained in previously conducted studies (Anyanwu et al., 2020a, Anyanwu et al., 2020b, Zhou et al., 2020a). On the other side, rats that were co-treated with HMM and Zn or Se or Zn + Se had less of heavy metals deposit in the cerebellum and cerebral cortex, nevertheless levels of Al, Pb, Mn and Hg in cerebellum and cerebral cortex were higher compared to controls; this could be partly due to e.g. Pb and Hg may use same transporter in intestine. Namely, Gunshin et al. showed that divalent metal transporter 1 (DMT1) could uptake broad range of metals, including Hg, Mn, Pb and Zn. So, as consequence all mentioned metals compete for same binding position (e.g. on the DMT1) and as less toxic metals will enter the bloodstream and form deposits in the CNS. We have to note that Zn may use DMT1 as a carrier but Zn has its own carrier in intestine and that is ZIP14 (Ohta and Ohba, 2020). This interference between the metals absorption at the level of intestine may only partly explain beneficial effects on Zn and Se to reduce toxicity induced by heavy metals.

HMM-induced neurotoxicity and antagonistic effects of various therapeutic procedures

Studies involving humans showed that exposure to heavy metals during pregnancy may be associated with anxiety symptoms in childbearing woman (Levin-Schwartz et al., 2022) and with neurodevelopmental disorders in infants (de Water et al., 2022). Zhou et al. conducted an in vivo study, in which authors treated newly pregnant rats with following concentrations of metal mixture (MM; Pb, Cd and Hg), 1-fold concentration (1 x MM), 5-fold concentration (5 x MM), 10-fold concentration (10 x MM) and controls; groups that were exposed

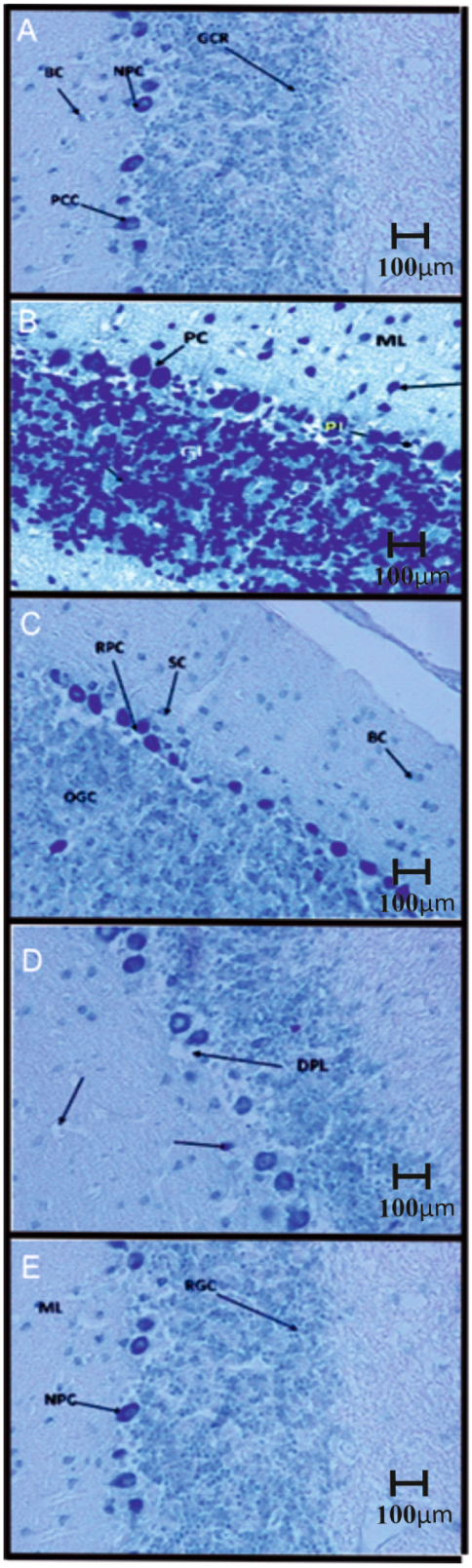

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Fig. 5. The cerebellum was examined under different conditions, as shown in Fig. 5. In Group 1 (control, deionized water; Fig. 5A), the molecular layer (ml) was observed, along with basket cells (BC), Purkinje layer (PL) and Purkinje cells (PC), as well as the granular layer (GL) with round cells (RC) at a magnification of 400x. In Group 2 (HMM only; Fig. 5B), the cerebellum exhibited vacuolation in the Purkinje layer with disorganized cell layers and neuronal darkening, also at 400x magnification. In Group 3 (HMM plus Zinc; Fig. 5C), the cerebellum showed an organized molecular layer (ml), Purkinje layer (PC), and granular layer (GC), along with regeneration of the cells. Group 4 (HMM plus Se; Fig. 5D) showed a photomicrograph section of the cerebellum with an organized cytoarchitecture of the molecular layer (ml), degenerated Purkinje layer (DPL), and granular layer (GL). Lastly, Group 5 (HMM plus Se plus Zn; Fig. 5E) demonstrated reorganized and regenerated layers at 400x magnification.

to MM were treated with MM from the day one of gestation till the end of lactation. After weaning (after lactation ended) 1 x MM and 5 x MM groups were treated with MM until they reached 83rd post-natal day (PND). On the other side, 10 x MM rats' pups were after weaning divided into three subgroups (first subgroup kept on ingesting MM in drinking water until 83rd post-natal day, second subgroup received distilled water without MM until 83rd PND while third group received enriched environmental intervention for the same period of time). The second and third subgroups of 10 x MM rats were treated with MM only during gestation and lactation, while afterwards were treated with water and enriched environment. Their results indicated that escape latency in the three exposed groups (5-fold concentration, 10-fold concentration, and 10-fold concentration rats that drank distilled water after birth) was significantly prolonged in contrast with that in the control (assessed by the Morris water maze test). Y Zhou et al. also noted that synapse density of CA1 hippocampal region significantly decreased in same three groups (5-fold concentration, 10-fold concentration, and 10-fold concentration rats that drank distilled water after birth) compared to the controls (Zhou et al., 2020b). Interestingly, they showed that enriched environmental intervention could mitigate some adverse effects induced by MM exposure. Branca et al. examined effects of Zn and Se in the Human neuroblastoma SH-SY5Y cell line that were exposed to 10 µM of cadmium for 24 h; they obtained that Se and Zn were able to prevent reduction in neuroblastoma cell viability, supress ROS production. Moreover, cells treated with Cd and Zn had lower levels of Casp3 compared to Cd treated cells (Branca et al., 2018). Although Branca et al. provided some evidence that Zn and Se could prevent neuronal-induced heavy metal toxicity, they examined only one metal cadmium in cell culture; in vitro studies lack complex organ environment effects. Anyanwu et al. aimed to evaluate whether *Costus afer* and Zn antagonize adverse effects of HMM (Cd, Pb and Hg) exposure in rats treated for 90 days; they showed Co-treatment with 2250 mg/kg of *Costus afer* or with *Costus afer* +Zn increased levels of IL-10, decreased concentrations of IL-6 and recovered antioxidant system in the frontal cortex of HMM treated animals (Anyanwu et al., 2020b). Nevertheless, Anyanwu et al. did not examine any transcriptional factors, also, it would be difficult to distinguish between beneficial effects of Zn and *Costus afer*.

All in all, available literature suggest that various metal mixtures or heavy metals alone may induce neurotoxic effects and protective procedures that can mitigate neuronal injury caused by HMM were enriched environmental intervention (Zhou et al., 2020a), *Costus afer* (Anyanwu et al., 2020a) or Zn (but this was in vitro study) (Branca et al., 2018) were evaluated; on the other side, it was shown that Zn and Se may attenuate injury caused by some metals, in other organs, such as kidney (Zhang et al., 2014, Babaknejad et al., 2016) and liver (El-Boshy et al., 2015), therefore, studied that examine protective effects of Zn and Se in various CNS are still lacking.

HMM exposure and Alzheimer disease

Studies have demonstrated that exposure to HMM may modulate the nuclear factor-kappa B (NF-kB) pathway, resulting in the production of pro-inflammatory cytokines and the upregulation of genes involved in oxidative stress(Chen and Shi, 2002). On the other hand, heavy metal mixtures can also affect the nuclear factor erythroid 2-related factor 2

(Nrf2) pathway, which plays a crucial role in cellular defence against oxidative stress(Buha and Baralić, 2021). Inflammation in the CNS lead to accumulation of ROS and MDA as consequence; and this study has demonstrated that MDA accumulated in cerebral cortex and cerebellum of rats because of Al, Hg, Mn and Hg exposure. Inflammation of the CNS promoted by innate immunity is observed in AD (Heneka et al., 2015). Also, we observed that exposure to the HMM potentiated activity of AChE, leading to decreased levels of acetylcholine while depression of acetylcholine levels in fibres originating from basal forebrain projecting to cerebral cortex is one of the main features of AD (H Ferreira-Vieira et al., 2016). Another important transcriptional factor examined in our study, Nrf2 interferes with main pathogenic processes in AD including Aβ and p-tau pathways (Osama et al., 2020). All the adverse effects of HMM could be antagonised by Zn and Se supplementation. Interestingly, individuals with AD have lower levels of Zn and Se in the blood (Socha et al., 2021). Whereas this study has not aimed to evaluate HMM exposure and onset of the AD given that AD is highly complex disorder, the present study has however demonstrated that HMM treatment may promote onset of AD. Perhaps a possible limitation of this study which emanated largely from the need to reduce the number of animals is the exclusion of animals administered only with zinc chloride, sodium selenite or zinc chloride plus sodium selenite. This omission will be addressed in future studies using relevant in vitro models. Furthermore it will be worthwhile to study the sexual dimorphic neuroprotective potentials of estrogen in heavy metal mixture mediated neurotoxicity (Petrovska et al., 2012).

Conclusion

Selenium and zinc exert neuroprotection via Nrf2/NF-kB signalling pathways against quaternary heavy metal mixture-induced (Al, Hg, Mn and Pb) impairments in female Sprague Dawley rats.

CRediT authorship contribution statement

Chinyere Dike, Data acquisition and analyses of data; Chinna N. Orish Data acquisition, analyses of data' manuscript drafting and conceptualization, Anthonet N. Ezejiofor, Data acquisition and analyses of data; Ana Cirovic and Aleksandar Cirovic, manuscript drafting; Bolaji Babatunde and Francis Sikoki, Supervision of Chinyere Dike; Orish E. Orisakwe analyses of data' manuscript drafting and conceptualization.

Declaration of Competing Interest

Authors declare no potential conflict of interest.

Data Availability

Data are available upon reasonable request from the corresponding author.

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