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ORIGINAL PAPER

Zn and Se protect toxic metal mixture-mediated memory deficit by activation of Nrf2-hmox-1 signaling in the hippocampus of female rats

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ABSTRACT

Introduction and aim. Heavy metals mediate neurotoxicity by altering some signaling pathways. This work investigated the effect of two nutritional elements Zn and Se on neurotoxicity caused by toxic metals.

Material and methods. Female Sprague Dawley rats (35) were divided in to 5 groups (7 rats/group), treated as follows: orally received deionized water (group I), heavy metals mixture HMM: 20 mg·kg $^{-1}$; 0.40 mg·kg $^{-1}$ of Hg; 0.56 mg·kg $^{-1}$ of Mn; and 35 mg·kg $^{-1}$ of Pb body weight; of Al (group II), HMM+Zn (zinc chloride; 0.80 mg·kg $^{-1}$) (group III), HMM+Se (sodium selenite: 1.50 mg·kg $^{-1}$) (group IV) and HMM+Zn+Se (group V). Before euthanasia of the rats, the Cincinnati Dry Maze, Barnes Maze, and Rotarod tests were performed. After euthanasia, the hippocampus was examined biochemically and histopathologically.

Results. Treatment with HMM induced inflammation with increased concentration of interleukin 6 and tumor necrosis factor α (30.70±6.65, p<0.02 pg/mg protein and 14.20±4.81 pg/mg protein, p<0.0001) respectively, compromised antioxidants levels, potentiated lipid peroxidation (higher malondialdehyde at 0.94±0.04 nmol/g tissue, p<0.0001 and nitric oxide at 3.89±0.16 nmol/g tissue, p<0.0001 levels), disrupted Nrf2 signaling pathway, potentiated acetylcholinesterase activity and induced mild pathohistological alterations in the rat hippocampus.

Conclusion. Supplementation with Zn and Se attenuated HMM mediated neurotoxicity.

Keywords. essential trace elements, heavy metal mixture, neurobehavioral testing, neurotoxicity, oxido-inflammation

Introduction

The hippocampus is one of the key regions of the limbic system. The hippocampus is broadly connected with other gray masses in the brain; through the Papez circuit, the hippocampus is connected with other relevant limbic regions in the medial limbic lobe and the hypothalamus. In addition, through the mesolimbic pathway, the hippocampus is linked to the ventral tegmental area,

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which is the main source of dopamine for the entire limbic system. Limbic system in general, is deeply involved in emotions, moreover, the hippocampus "decides" which new information will enter a long-term memory and which newly acquired information will get forgotten. Therefore, any potential damage to the hippocampus could lead to memory impairment and humans are continuously exposed throughout life to various metals such aluminum (Al^{3+}), lead (Pb^{2+}), mercury (Hg^{2+}) and manganese (Mn^{2+}) which have been shown to be neurotoxic.[1-5] In most developing nations such as Nigeria and other sub-Saharan African countries, various anthropogenic activities including e-waste have negatively impacted the environment with studies showing contamination of potable water and food, etc. with heavy metals (Pb, Hg, Al, and Mn).[6-11]

Aluminum lurks in infant milk formulas,[12] vaccines[13] and various foodstuffs.[14] Jin et al. in their study showed that grains and vegetables are contaminated with lead, whereas tea was measured with highest lead levels (1.937 mg/kg).[15] Fish and seafood represent major source of mercury; moreover, Feingold et al, showed that regions such as Madre de Dios showed that 37.7% of the people living in mentioned regions of Peru exceed the World Health Organization (WHO) recommended mercury permissible level (>2.2 μg/g).[16] Such high levels of mercury were linked with the consumption of fish. It is reasonable to speculate that the majority of mentioned foodstuffs are used daily, i.e. all meals contain them and consequently humans are exposed to heavy metals via gastrointestinal route throughout their entire life.

All studies conducted so far showed that heavy metals-induced neurotoxicity is provoked by oxidative stress, increased lipid peroxidation and diminution of antioxidants.[2] Bittencourt et al. showed that treatment with Al *per os* in rats mediated changes in protein expression and decreased cellular density in some regions of the hippocampal regions such as the cornu ammonis 1 (CA1), CA3 and dentate gyrus;[4] nevertheless, majority of studies that examined adverse effects of heavy metals were focused on a single metals exposure.[2,4] Limitations of such studies are that individuals are exposed to cocktail of metal mixtures on the daily basis, and to a single metal; next, particular combinations of metal mixtures have synergistic hazardous effects such as Pb and Hg.[17] On the other side what remains unknown is which intracellular signaling pathways is dysregulated by heavy metals and as result of ROS production and antioxidant depletion appear.[18,19]

Deficiencies in Se and Zn are linked with decreased activities of some antioxidants; also metal transporter 1 (DMT1), a protein largely expressed in the proximal duodenum, could uptake Zn and Pb and since both metals compete for the same binding place, as a consequence Zn could decrease the bioavailability of heavy metals and as consequently provide some protective effects.[20]

Aim

In view of these, this study has evaluated (a) modulation of hippocampal apoptosis, intracellular glutathione stores, and Nrf2-hmox-1 signaling in rats by the quaternary toxic metal mixture (Pb, Hg, Mn; and Al) and (b) potential neuroprotective effects of Zn and Se against HMM-induced toxicity, after oral exposure.

Materials and methods

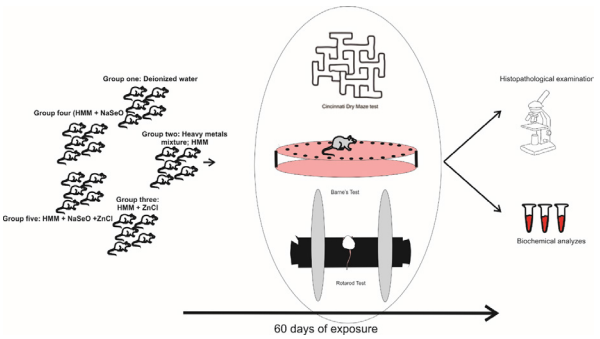

Fig. 1. Schematic representation of methodological approach

Animals' husbandry and treatments

The animal husbandry and treatment protocol adapted our previous protocol reported elsewhere.[21] Briefly, we purchased 35 female Sprague Dawley rats (6-8 weeks of age) from the Faculty of Pharmacy, Animal House, University of Port Harcourt (Animal House), Rivers State, Nigeria. Sprague-Dawley rats were kept at an ambient temperature of 25±2°C with 12-h light/dark cycles. Before the beginning of this study, the Sprague Daley rats were adapted for fourteen days. The rats were placed in five groups (7 per group). The experiment lasted 60 days and treatments were performed by an oral route. Rats in group 1 or controls received deionized water. Group 2 received Pb, Hg, Mn and Al (20 $mg \cdot kg^{-1}$ [22] Pb; 0.40 $mg \cdot kg^{-1}$ of Hg [22]; 0.56 $mg \cdot kg^{-1}$ of Mn; and 35 $mg \cdot kg^{-1}$; of Al [23-25] body weight as a mixture (Sigma Aldrich WGK Germany). Groups 3,4 and 5 received HMM and in addition to 0.80 mg/kg $ZnCl_2$[26], sodium selenite 1.50 mg/kg Na_2SeO_3;[27] and both $ZnCl_2$ and Na_2SeO_3, respectively. Randomization and blinding were used to reduce bias. Doses that mimicked environmental availability, as previously reported in our lab, were used.[28] The metal mixture was rated low,[22] and even lower doses were used elsewhere.[30-31]

Feed and water intakes were recorded every day. The weight of the rats was recorded every week. Pentobarbital (50 mg/kg, ip) was used as an anesthesia agent in sacrificing the animals. The hippocampus of each rat was dissected, separated as previously described in our

laboratory.[26] This was used for both biomarkers and determination of Pb, Hg, Mn and Al.

The protocol was approved by the Research Ethics Committee of the University of Port Harcourt (UPH / PUTOR / REC/12). The study was in line with the "Guide for the Care of Laboratory Animals" ratified by the National Academy of Science (NAS). The rats received standard food and deionized water without restriction.

Cincinnati Dry Maze Test

Cincinnati Maze was employed in the evaluation of egocentric navigation, learning, and memory, as described previously.[32]

The rat was kept at the door of the box and the entrance closed, and allowed it to navigate through the box to the exit, the time it took the rat to navigate its way from the entrance point to the exit point is recorded. The rat was timed for 300 seconds and was expected to find its way to the exit point within that time, the shorter the time it completed its task, the more intact the memory. If the rat cannot find its way to the exit within 5 minutes, the task was assumed to be incomplete.

Barnes maze

Furthermore, Barnes maze is a dry land maze used as a test for visual-spatial learning and memory (hippocampal damage).[33]

The rat was put on the Barnes apparatus and observed for 300 seconds until it located the safe spot. The time to locate the safe spot by the rat was recorded. If the rat was unable to identify the safe area, 0 or 1 is reported.

Rotarod test

This used an accelerating rotarod as described by Jacquez and co-workers.[34] This was done on a rotarod to assess the riding time or endurance, grip strength, motor balance, and coordination of the animals and check for cerebellar damage. This includes a task performed on an accelerating rotarod to determine how long an animal can sustain walking on a rolling rod. On the rotarod, rats were put on a rotating drum (30 mm diameter, 85-mm width). It assesses the rat's capacity to feel equilibrium by measuring how long it lasts on a rotating drum. Over the course of 5 minutes, the rod's velocity increased from 4–20 revolution per minute, and the time to fall was measured. The rats were given at least some time to rest between trials to reduce stress and exhaustion.

Enzyme-linked immunosorbent assay (ELISA) analysis

The assay that determined the levels of cytokines, transcription factors, acetylcholinesterase (AChE), caspase an apoptotic marker in the hippocampus, was previously described in another study.[35]

Oxidative stress markers

The glutathione peroxidase (GPx) was carried out using Paglia and Valentine method,[36] while reduced glutathione (GSH) was Marklund and Marklund method.[37] The method of Habig et al. was to determine glutathione-S-transferase (GST).[37] The activity of superoxide dismutase (SOD) activity was assayed using the method of Jollow et al.[38] Catalase (CAT) activity was estimated by monitoring the rate of breakdown of H_2O_2 at 240 nm according to Aebi's method.[39]

Lipid peroxidation was determined as reactive substances with thiobarbituric acid by the adaptation of Ester Bauer and Cheeseman method at 532 nm. The Griess reaction technique was adapted in the analysis of nitric oxide (NO).[41] In all UV-vis spectroscopy (Agilent Technologies, Cary 8454, UV–visible spectrophotometer, Santa Clara, California, USA) was used.

Estimation of Al, Mn, Pb and Hg levels in the hippocampus

The hippocampal levels of Al, Mn, Pb, and Hg were determined using the atomic absorption spectrometer.[42]

Histopathological examination

Rats were sacrificed one week after the behavioral test. Histopathological slides were prepared and examined as previously described in our lab.[26]

Statistical analysis

Data were expressed as mean±SD. Microsoft Xlstat 2014 was used in performing ANOVA and Tukey multiple comparison pairwise tests all significant differences were at a $p<0.05$.

Results

Absolute and relative weight of the hippocampus; feed and fluid intakes of female Sprague-Dawley rats after HMM (Pb, Mn, Hg, and Al)

After the end of the experiment, the control group 1 had higher body weight compared to the HMM, HMM+Zn, HMM+Se and HMM+Zn+Se groups ($p<0.05$); moreover, HMM and HMM+Zn animals had a higher body weight compared to HMM+Se and HMM+Zn+Se ($p<0.05$). On the other hand, feed and water intakes were similar between groups ($p<0.05$). Also, the weight of the hippocampus did not vary between the examined groups ($p<0.05$). The HMM-exposure rats had significantly higher hippocampus weight compared to either controls ($p<0.05$) or the HMM+Zn, HMM+Se and HMM +Zn+Se groups ($p<0.05$) (Table 1).

Accumulation of heavy metals in the hippocampus

Effect of essential elements on heavy metal accumulation in the hippocampus

The effect of the combination of Zn alone, Se alone, and Zn+Se on heavy metals accumulation in the hip-

pocampus is shown in Figure 2. We found the following percentage of reduction 44.53% in HMM+Zn, 42.34 in HMM+Se, and 51.82 in HMM+Zn+Se. The HMM+Zn animals had a mean Hg value of 0.092±0.002 mg/kg, HMM+Se had a mean value of 0.089±0.002 mg/kg and HMM+Zn+Se had a mean value of 0.080±0.001 mg/kg (Fig. 2A).

Table 1. Effect of Zn and Se on body weight, absolute and relative weight of hippocampus feed and fluid intakes of female Sprague Dawley rats after exposure to heavy metal mixtures (Pb, Mn, Hg, and Al)

Treatment	Absolute (g)	Relative (%)	Body weight (g)	Feed intake (g)	Fluid intake (mL)
Control	0.12±0.08^a	7.04	200.00 ±14.14	154.10 ±23.42^a	242.98 ±35.36^a
HMM only	0.10±0.01^a	5.28	180.50 ±0.71^b	148.27 ±16.42^a	210.07 ±33.46^a
HMM+Zn (0.8 mg/kg)	0.10±0.01^a	6.06	162.50 ±12.02^b	147.94 ±22.13^a	195.62 ±43.20^a
HMM+Se (1.5 mg/kg)	0.13±0.08^a	8.02	155.50 ±0.71^c	127.63 ±35.47^a	209.74 ±51.27^a
HMM+Zn +Se	0.10±0.03^a	6.25	155.00 ±7.07^c	127.82 ±29.85^a	203.85 ±53.26^a

* mean±SD, dissimilar characters a, b, c, d denote a significant difference between the means at p<0.05 but when they are the same there is no significant difference (n=5)

The following concentrations of Pb, 0.448±0.006 mg/kg, 0.511±0.009 mg/kg and 0.410±0.003 mg/kg for HMM+Zn, HMM+Se and HMM+Zn+Se rats, respectively, were observed. The essential elements show a percentage reduction of 38.38 in HMM+Zn rats, while HMM+Se rats showed a percentage reduction of 29.78 and HMM+Zn+Se rats showed a percentage reduction of 43.60 (Fig. 2B).

A mean concentration of Mn in HMM+Zn rats was 0.038±0.001 mg/kg, 0.040±0.003 mg/kg in HMM+Se rats, and 0.033±0 mg/kg in HMM+Zn+Se rats. The percentage reduction in HMM +Zn was 48.02, HMM+Se showed a percentage reduction of 49.72 and HMM+Zn+Se showed a percentage reduction of 59.80 (Fig. 2C).

The effect of the combination of Zn only, Se only, and Zn+Se caused significant decrease (p<0.05) in the level of aluminum in the hippocampus (Fig. 2D). HMM+Zn had a mean aluminum concentration of 0.407±0.002 mg/kg, HMM+Se had a mean value of 0.539±0.007 mg/kg and HMM+Zn +Se had a mean value of 0.388±0.007 mg/kg. HMM+Zn had a percentage reduction of 57.52, HMM+Se had a percentage reduction of 43.67 and HMM+Zn+Se had a percentage reduction of 59.46 due to treatment with essential trace elements (Fig. 2D).

Effects of HMM (Pb, Mn, Hg and exposure to Al) and Zn and Se on antioxidants (SOD, GPx, CAT, GSH) and MDA and NO levels in the hippocampus of female Sprague Dawley rats

SOD activity decreased significantly in rats exposed to HMM in comparison to controls (p<0.05). The SOD activity was the same as in the HMM and HM-

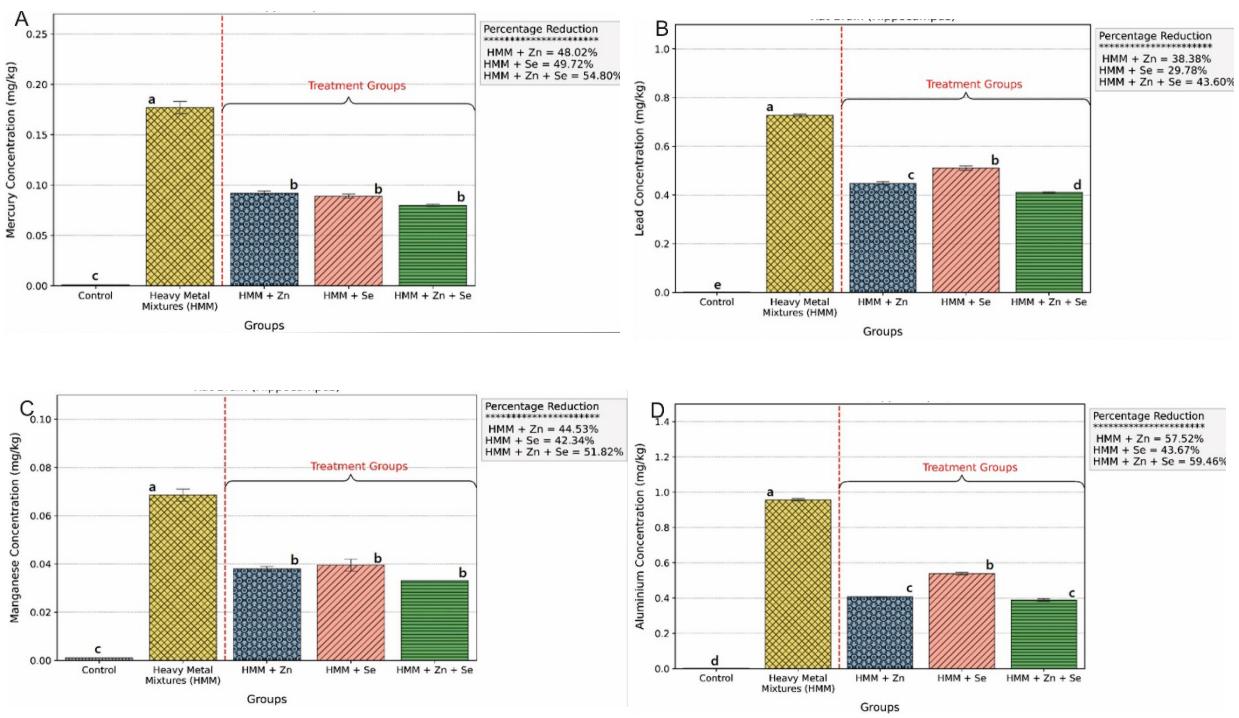

Fig. 2. Graphical representation of heavy metal accumulation in the hippocampus – A: mercury, B: lead, C: manganese and D: aluminum (mean±SD, dissimilar characters a, b, c, d denote significant difference between the means at p<0.05 but when they are the same there is no significant difference (n=5)

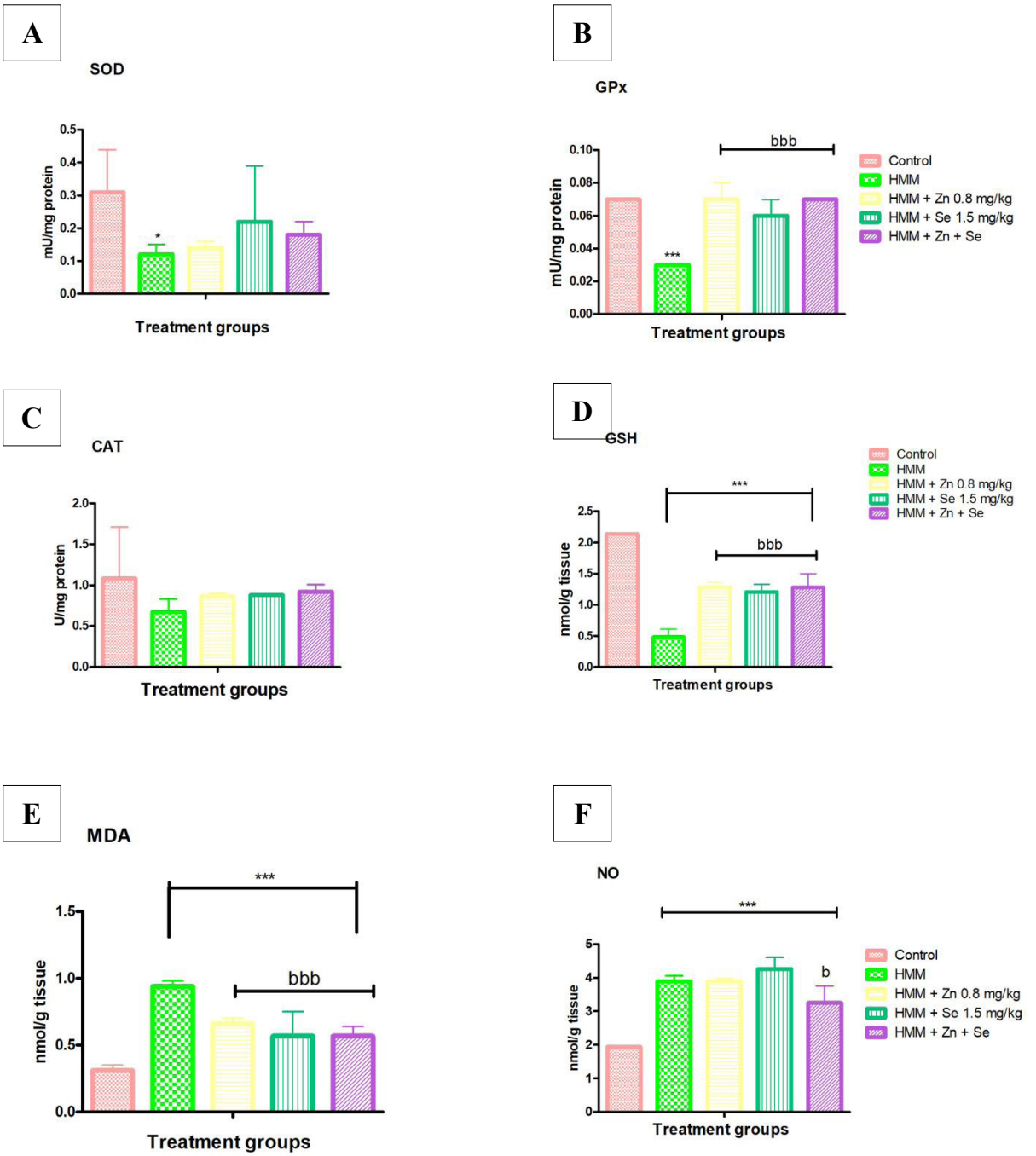

Fig 3. Effect of essential elements on antioxidants (SOD, GPx, CAT, GSH) and MDA and NO levels in the hippocampus of female Sprague-Dawley rats after HMM (Pb, Mn, Hg, and Al) (*** – significantly different compared to control at p<0.001; ^{bbb} – significantly different compared to HMM at p<0.001)

M+Zn groups of animals; however, HMM+Se and HMM+Zn+Se animals had the SOD activity like unexposed controls rats and significantly higher than HMM exposed rats (Fig. 3A). GPx levels did not vary between controls, HMM+Zn, HMM+Se and HMM+Zn+Se groups (p> 0.05), while the controls, HMM+Zn, HMM+Se and HMM+Zn+Se groups (p>0.05), while all four mentioned groups (controls, HMM+Zn, HMM+Se, and HMM+Zn+Se) had higher (p<0.05) levels compared to the HMM treated rats (Fig. 3B). CAT activity was similar (p>0.05) between the five examined groups of animals (Fig. 3C). We observed a significant decrease in GSH levels in the HMM treated animals compared to controls (p<0.05), while Se and Zn supplementation significantly but incompletely recovered GSH levels; that is, all three groups co-treated with essential trace metals (HMM+Zn, HMM+Se and HMM+Zn+Se) had higher GSH levels higher than HMM exposed group (p<0.05)

and significantly lower than unexposed controls rats ($p<0.05$) (Fig. 3D).

HMM-treated rats had higher levels of MDA compared to the unexposed controls rats ($p<0.05$), moreover, all three groups co-treated with essential trace metals (HMM+Zn, HMM+Se and HMM+Zn+Se) had MDA levels higher than controls ($p<0.05$) and significantly lower ($p<0.05$) than HMM-exposed animals (Fig. 3E). In Figure 3F, HMM, HMM+Zn, HMM+Se and HMM+Zn+Se had similar NO levels while ($p>0.05$). Unexposed controls rats had lower ($p<0.05$) NO levels ($p <0.05$) compared to groups treated with heavy metals treated groups (HMM, HMM+Zn, HMM+Se and HMM+Zn+Se).

Effect of Zn and Se on levels of inflammatory cytokines (IL-6 and TNF-α), transcription factors (Nrf2 and NF-kB) and caspase 3 in the hippocampus of female Sprague Dawley rats after exposure to HMM (Pb, Mn, Hg, and Al)

There were significantly higher levels of IL-6 and TNF-α in rats exposed to HMM compared to controls not exposed (Fig. 4A and 4B). Regarding IL-6, we obtained that all three groups co-treated with HMM and Zn, Se and Zn+Se had similar levels as controls did ($p>0.05$), while on the other side, all three groups co-treated with HMM and Zn, Se had significantly ($p<0.05$) lower levels of TNF-α compared to the group treated only treated group.

The Nrf2 level decreased significantly in animals exposed to HMM only when compared with the control, HMM+Zn, HMM+Se and HMM+Zn+Se (Fig. 4C). There was a significant increase in Caspase-3 (Casp-3) levels in animals exposed to HMM compared to the unexposed control (Fig. 4D). HMM+Zn, HMM+Se and HMM+Zn+Se had significantly lower Casp-3 levels than exposed rats (Fig. 4E).

Effect of Essential Elements Zn and Se on Cincinnati Dry Maze Test, Barnes and Rotarod Performance Tests

Cincinnati Dry Maze Test

Figure 5A represents the Cincinnati Dry Maze performance test of rats exposed to HMM treated with Zn and Se. The unexposed control group showed significantly lower escape latency in comparison to HMM exposed rats. The HMM exposed rats showed significantly ($p<0.05$) higher escape latency than the unexposed control. The escape latency of the groups co-treated with essential elements decreased significantly compared with the HMM only group.

Barnes test

The Barnes performance test is shown in Figure 5B. The unexposed group had a lower escape latency time for locating the hole when compared with the HMM only exposed group. The escape latency of the groups treated with Zn alone,, Se only and the combination of Zn plus Se was significantly ($p<0.05$) lower than the group with HMM only group.

Rotarod test

The performance test is shown in Figure 5C. Animals treated with essential elements in the Rotarod test are shown in Figure 4C. Rats that received deionized water (control group) had significantly ($p<0.05$) longer fall

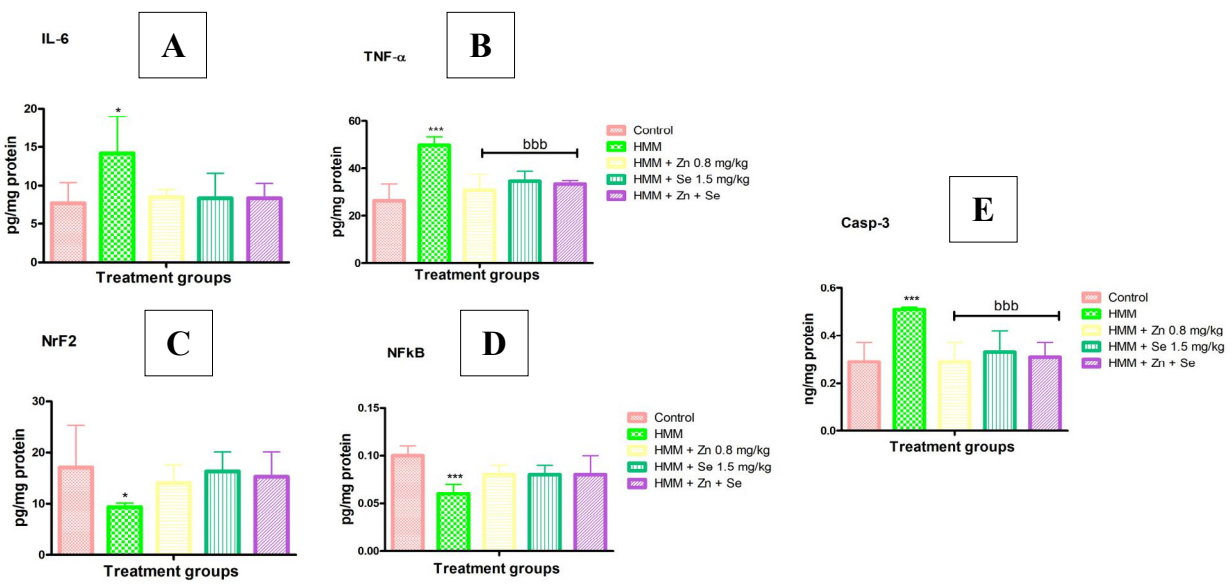

Fig. 4. Effect of Zn and Se on the pro-inflammatory cytokines IL-6, TNF-α, transcription factors (Nrf2; NF-kB) and caspase 3 in the Hippocampus of female Sprague Dawley rats after exposure to HMM (Pb, Mn, Hg, and Al) (* – significantly different compared to control at $p<0.05$; *** – significantly different compared to control at $p<0.001$; ^{bbb} – significantly different compared to HMM at $p<0.001$)

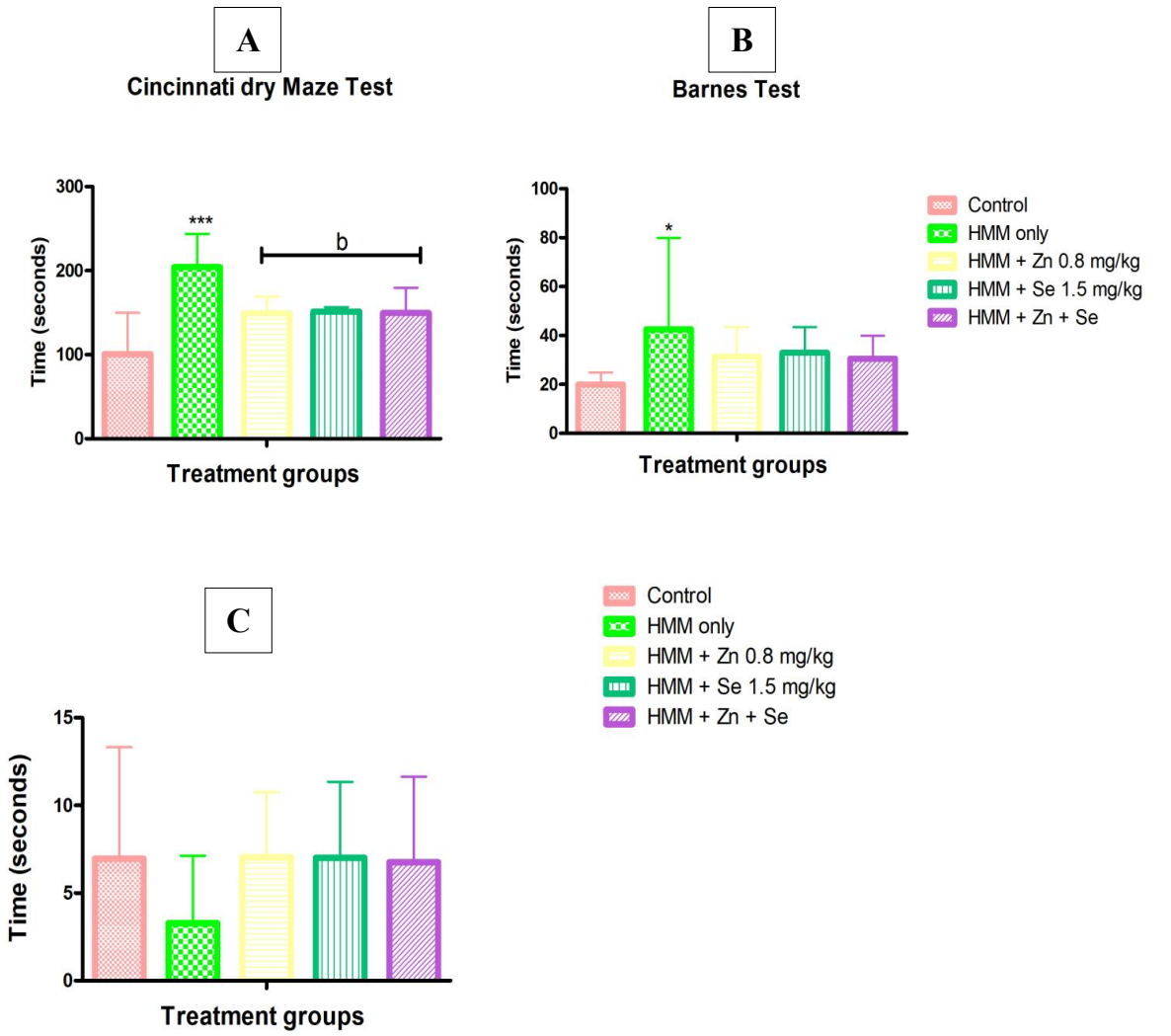

Fig. 5. Effect of Zn, Se on Cincinnati dry Maze, Barnes and Rotarod tests (time sec, * significantly different compared to control at $p<0.05$, *** – significantly different compared to control at $p<0.001$, * – significantly different compared to HMM at $p<0.05$)

time from the rotating wheels/rod of the rotarod than all groups when compared with the HMM-only group. The HMM only group had significantly ($p<0.05$) shorter latency of fall. The latency of fall in the HMM plus Zn, HMM plus Se, and HMM plus Zn plus Se were longer than that of the HMM only group.

Effect of essential trace element on AChE activity female Sprague Dawley rats after HMM (Pb, Mn, Hg and Al) exposure

We found the following levels of AChE, 95±7.07 µmol/mL, 285±7.07 µmol/mL, 110±0.00, 135±49.50 µmol/mL, 170.5±14.85 µmol/mL for control, HMM, HMM+Zn, HMM+Se and HMM+Zn+Se, respectively. There was a significant increase in animals exposed to AChE activity in HMM compared with other groups.

Histopathological examination

The hippocampus of rats exposed to only deionized water showed a regular architecture of the CA1 and CA3 regions, the dentate gyrus (DG), and the subicular cortex (subiculum); while HMM treated rats had severe neuronal cell loss of neurons with associated diffuse chromatolysis. The number and morphology were similar in HMM+Zn as in controls, while features of HMM+Se neurons showed minimal darkening. The hippocampus of animals exposed to HMM+Zn+Se showed normal neuronal density with dysplasia in the CA2 region (Fig. 6).

Discussion

In this study, low environmental levels of heavy metals have been shown to induce inflammation, deplete antioxidants, disruption of the Nrf2 signaling pathway, potentiated acetylcholinesterase (AChE) activity in the hippocampus with associated memory impairment, and mild histological alterations. The wide range of parameters examined (IL-6, Nrf2, Casp-3, GPx, CAT, and NO) which were reversed after supplementation with essential trace elements tend to reflect the significance of Zn and Se in neuroprotection. There was an increase in brain

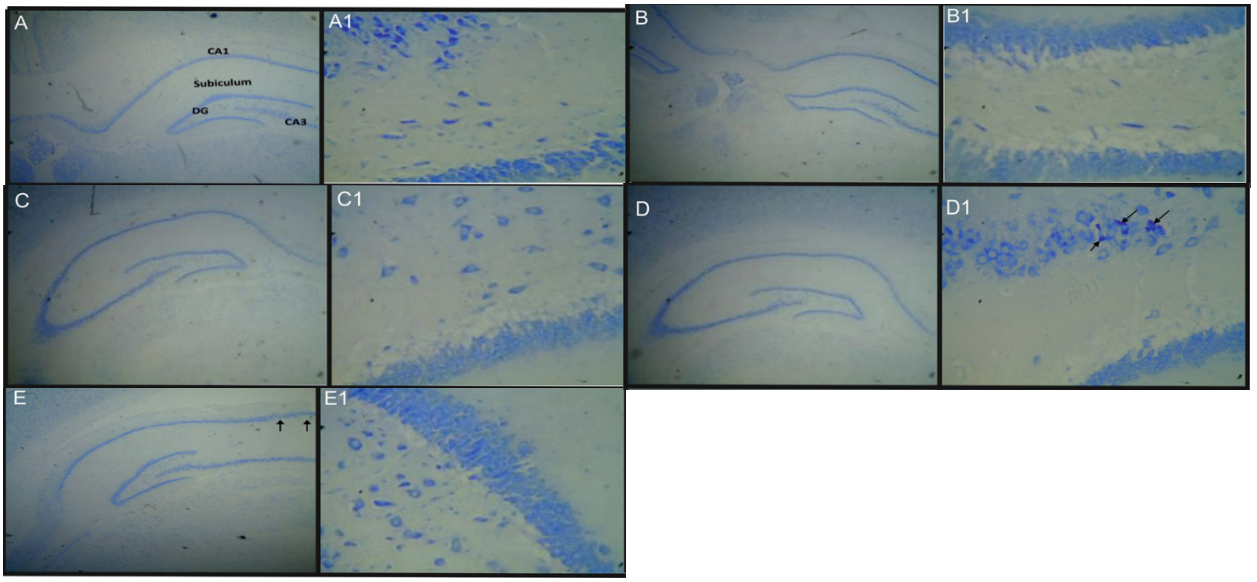

Fig. 6. A: Photomicrograph (toluidine X40) and 3A1: normal hippocampus (toluidine X400) exposed to only deionized water showing the CA1 and CA3 regions, the dentate gyrus (DG) and the subicular cortex (subiculum), B: hippocampus (toluidine X40) exposed to only HMM showing mild loss of neuronal cells, B1: hippocampus (toluidine X400) exposed to only HMM showing severe loss of neuronal cells with an associated diffuse chromatolysis; C: hippocampus, normal (toluidine blue X40) exposed to HMM+Zn, C1: hippocampus, normal (toluidine blue X40) exposed to HMM+Zn, D: hippocampus (toluidine blue X40) exposed to HMM+Se showing minimal neuronal darkening, D1: hippocampus (toluidine blue X400) exposed to HMM+Se showing multiple foci of necrosis (arrows), E: hippocampus (toluidine blue X40) exposed to HMM+Zn+Se showing normal neuronal density with dysplasia at CA2 region (arrow), E1: hippocampus (toluidine blue X400) exposed to HMM+Zn+Se showing normal cellularity

weight in HMM exposed rats, highly likely due to edema induced by inflammation; similar findings regarding increased brain weight are observed in similar studies. Some studies have shown that HMM heavy metal mixture decreased body weight gain, increased relative organ weight, with brain edema, and induction of apoptosis.[43-44]

As expected, all the heavy metals accumulated in abundance in the hippocampus of treated rats; Interestingly, there were studies that showed that some of the metals used in this study, eg aluminum, are found to a AD patients in greater extent.[45-46] Histology s of the hippocampus in Bittencourt et al., study, where authors treated male Wistar rats with aluminum 8.3 mg/kg for two months, revealed a decrease in the thickness of hippocampal cell bodies and the dentate gyrus; also, by using water maze test, they found that used low levels of Al may induce memory deficit of the rats.[4] Other *in vivo* studies showed that single treatment with other metals used in our study, e.g. lead also may lead to disturbances in neurobehavioral testing (radial arm maze).[47]

It is well known that iron is an integral part of CAT, in other words, its antioxidant activity is iron dependent.[48] and the observed presence of heavy metals (Al, Pb, Mn and Hg) led to decreased levels of CAT; There are no ways to eliminate excess of iron from the centrals nervous system (CNS), so iron that originated from damaged CAT would likely accumulate in the CNS, or actually in the hippocampus in this case. When cellular iron levels overcome the cellular ability to bind it, iron starts to exert neurotoxicity in a reaction known as ferroptosis.[49]

Karri et al. conducted an in vitro study in which authors exposed HT-22 cell line (originate from immortalized mouse hippocampal neuronal precursor cells) to single heavy metals (Cd, Pb, Hg and As) and to various combination of them[50] for eight days. They found that all metals examined, as a single exposure, decreased cell vitality and provoked apoptosis in a dose-dependent manner and also noted that among the four heavy metals, not all of them have similar potential to induce neurotoxicity (e.g., mercury appeared to induce apoptosis and decrease neuronal viability at lower doses than Pb and Cd). Interestingly, Karri et al. also noted that some combinations of metals have synergistic adverse effects (eg Pb and Hg) posing a significant hazard to hippocampal precursors, while on the other side some combinations such as Cd and As had antagonistic effects,[50] i.e., induce cell damage to a lesser extent compared to single exposure (either Cd or As). This study raises the significance of our study since in our mixture, Pb and Hg were used. Jia et al. divided pregnant 8 week old Sprague-Dawley rats to four groups and treated them with metal mixture (MM; metal mixture; Cd, Pb and Hg) *per os,* following pattern 1 x metal mixtures (MM), 5 x MM, 10 x MM and controls;[51]

animals were exposed to MM until the end of weaning (post-natal day 23). They found that 1 x MM, 5 x MM pups did not experience any significant neurotoxicity while 10 x MM showed suboptimal search strategies which underlies the early cognitive deficits; Transmission electron microscope revealed severe neuronal loss in CA1 and CA3 areas coupled with reduced organelles and mitochondrial swelling.[51] In the similar experiment, which had the same groups and exposure pattern, but lasted until 83 postnatal day (PND), ultrastructure analyzes revealed that 10 x MM induced reduction in synapse density in the CA1 region.[31] Both studies examined effects of metals mixtures in the hippocampus, involved rats' puppies and examined morphological and cognitive aspects of metal toxicity while pathophysiological mechanisms on which metal induced neurotoxicity in the hippocampus is based were not evaluated.

Behavioral effects

The ability to advantageously maneuver through the environment in search of either feed and avert danger deemed central to the survival of most animals is mostly dependent on learning and recalling different locations and is encoded in the brain by two systems:[52] allocentric navigation and egocentric navigation.[53,52]

Exposure to heavy metals caused learning and memory deficits in animals with longer escape latency,[31,54] It is believed that this must be due to heavy metal-mediated hippocampal impairments. Heavy metals are known to alter hippocampal N-methyl-D-aspartate NMDA and muscarinic cholinergic receptors involved in spatial learning.[31,55,56]

With respect to the Barnes test, the HMM caused significantly longer time to locate the hole than the control group that received only deionized; this is indicative of spatial memory and learning ability loss mediated by the heavy metal mixture. Supplementation with Zn alone, Se only and Zn plus Se combination significantly improved the latency in locating the hole than the HMM only exposed group suggestive of improved spatial memory and learning ability. Although unlike the cerebellar areas of the brain, the hippocampus is often not associated with balance or motor functions, there are more recent facts that tend to implicate the hippocampus in maintaining balance.[57-58]

There were distortions in motor coordination, as shown in this study. In the Rotarod test, the latency is measured when the rats fall from the rotating cylinder. Diverse animal models of neuropathologies such as cerebellar ataxia, Parkinsonism, stroke show poor performance on the Rotarod test.[59-60] This work has shown that rats treated with HMM only showed loss of balance in the Rotarod test.[59-60] Rats treated with HMM only expended less time than the control group on the rotating cylinder, whereas rats that received zinc, selenium, and zinc and selenium combination expended more time on the rotating cylinder compared to the rats treated with HMM only group. The Rotarod test is characterized by exercise with the production of free radicals that overwhelm the rat's antioxidant machinery of the rat, culminating in the destruction of the cell membrane and skeletal muscle damage.[61-62] This cascade is even exacerbated in the HMM-only exposed rats and may have accounted for the reduced latency required for rats to fall from the rotating cylinder. It has been opined that ROS mediated cellular injury may be annulled by skeletal muscle antioxidant enzymes.[61] Exaggerated levels of ROS following HMM can alter intracellular signaling pathways by altering cellular functions.[63] Since selenium is a known antioxidant enhancer, this may explain the increased latency taken for rats to fall from the rotating cylinder seen in groups treated with essential trace elements.[61]

One of the most striking findings in our study was that HMM-induced disruption and decrease in Nrf2. Nrf2 attenuates oxidative and xenobiotic stress.[64] Increased oxidative stress mediated by accelerated ROS generation has been implicated in neurogenerative diseases.[65-68] Nrf2 also activates genes that encode antioxidant apparatus in a cell, including SOD, CAT, and GPx[69] and depletion of Nrf2, observed in HMM exposure rats, led to downregulation of antioxidants and afterwards accumulation of ROS became inevitable. When ROS accumulates in abundance, cells damage may appear and injured cells can stimulate innate immunity.[70] NF-kB is a key regulator of innate immunity and could possibly affect TNF-α levels, nevertheless, we did not detect disturbances in the NF-kB levels between the group exposed to HMM and controls. So, following axis, exposure to HMM - Nrf2 pathway disruption - ROS antioxidants depletion – generation - neuroinflammation could partly explain HMM-induced neurotoxicity. Today, the Nrf2 signaling pathway and its link with neurodegenerative disorders, as well as a potential therapeutic approach, attract the attention of the scientific community more than ever.[71-76] Nrf2 decreases the levels of α-synuclein aggregates that have been implicated in (PD).[77,72] Sarlette et al. examined the expression of Nrf2 levels in the primary motor cortex and spinal cord of 5 individuals with diagnosed ALS) and five controls and found lower levels of Nrf2 in subjects with ALS for both examined locations.[78] Down-regulation of the Nrf2 is hallmark of aging and neurodegenerative disease.[79] Von Otter et al. reported association between one NFE2L2 haplotype (GAAAA) with faster AD disease progression.[80] All mentioned neurodegenerative disorders are highly complex and still insufficiently evaluated and although we did not aim to examine contributing effects of heavy metals in onset of various neurodegenerative disorders some similarities are doubtless. Interestingly we found in our study that cholinergic disruption appeared due to increased AChE activity and low levels of acetylcholine in the basal fore-

brain correlates with decreased walking speed among individuals with PD[81] and AD.[82]

To counteract oxidative stress, a plausible modulator of cellular defense Nrf2 and Keap1 serves as a usual intrinsic defense system.[83] Exposure to heavy metals and metalloids and metals through diet, drinking water, air and diverse anthropogenic activities is linked to production of ROS in many organs, including the brain by both direct and indirect means.[84] Nrf2 signaling sustains antioxidant balance and can have two roles depending on the biological setting. Nrf2 is protective against metal-induced toxicity. The absence of female reproductive hormones, which would have offered more information to support the earlier assertion that even when female rats are used in neuroscience experiments without regard to the estrous cycle stage, their data are not more variable than those of male rats, may be considered a likely weakness of this study.[85]

Taken together, HMM-induced neurotoxicity mimics the cellular basis of neurodegenerative diseases in many ways: i) increased brain accumulation of heavy metals (observed in both), ii) ROS-mediated cellular damage, iii) disruption of the Nrf2-signaling pathway, and iv) low levels of acetylcholine. These pathological findings were attenuated by Zn and Se supplementations.

Metals (Al, Pb, Hg, and Mn)-induced neuronal toxicity in the hippocampus may be mediated by ROS accumulation, antioxidant depletion, Nrf2 pathway disruption, and increased activity of AChE. Importantly, most of altered parameters were reversed when animals were co-treated with Zn and Se, reflecting neuroprotective effects.

Declarations

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Author contributions

Conceptualization, C.N.O. and O.E.O..; Methodology, C.S.D., C.N.O, A.N.E.; Validation, C.N.O. and O.E.O.; Formal Analysis, C.S.D., C.N.O. and A.N.E.; Investigation, C.S.D., C.N.O. and A.N.E.; Data Curation, C.S.D., C.N.O. and A.N.E.; Writing – Original Draft Preparation, C.N.O., Ai.C., An.C., T.C.U. and O.E.O.; Writing – Review & Editing, Ai.C., An.C., T.C.U. and O.E.O.; Visualization, O.E.O.; Supervision, C.N.O., A.N.E. and O.E.O.

Conflicts of interest

Authors confirm that there was no conflict of interest.

Data availability

All data have been provided.

Ethics approval

Ethical approval was given by the University of Port Harcourt Research Ethics Committee (UPH/PUTOR/REC/12).

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