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Mercury Exposure and Post Exposure Recovery of the Cerebellar Cortex and Hippocampus of Adult Wistar Rats

[1]Sadeeq AA, [1]Bauchi ZM, Tanko M, Timbuak JA, Mukhtar AI, [1]Fatika AR, [1]Abdullahi A,[2]Muhammad Z, [3,4]El-ladan IS, [5]Rilwan BH

[1]Department of Human Anatomy, Neuroscience unit, Faculty of Basic Medical Sciences, Ahmadu Bello University, Zaria. Nigeria.

[2]Department of Human Anatomy, Faculty of Basic Medical sciences, Damaturu, Yobe state University. Nigeria.

[3]Department of Human Anatomy, Faculty of Basic Medical Sciences, Umaru Musa Yar'addua University, Katsina, Nigeria.

[4]Department of Forensic Sciences, School Of Basic And Applied Sciences, Golgotias University, Greater Noida, Uttar Pradesh, India.

[5]Department of Human Anatomy, Faculty of Basic Medical Sciences, Kaduna state University, Kaduna, Nigeria.

Corresponding author: Sadeeq AA
E-mail: aasadeequ@gmail.com; +2348038250628

ABSTRACT

Global environmental toxicants including Mercury (Hg) has potential health hazards to both human and animals. The studies investigate role of mercury exposure (ME) and mercury post exposure recovery (MPER) on grip strength and memory of adult Wistar rats. Eighteen adult Wistar rats were divided into three groups (I, II and III) with six rats per group. Group I as control, administered normal saline. Groups II and III were treated with doses of Hg at 12.45mg/kg and 24.9mg/kg respectively for twelve consecutive days. Motor balance test using grip strength test and novel objects recognition test for short term memory were conducted during ME and MPER phase. Animals were further divided into subgroups Ia&Ib, IIa&IIb, and IIIa&IIIb of three rats per subgroup. Rats in subgroup 'b' were allowed for 12 days MPER period while animals in subgroup a' were anesthetized. Brain tissues obtained were fixed in a Bouin's fluid for routine histological tissue processing. Motor balance coordination test indicates significant ($p<0.01$)decrease in time taken to release the string wire. The mean time in post exposure recovery period increased significantly ($p<0.01$). Short term memory test revealed a significant decrease and increase ($p<0.001$) for familial and novel objects during exposure and post exposure respectively. Histopathological observations reveal neurodegenerative changes in the cerebellar cortex and hippocampus that include pyknosis, karyorrhex is and karyolysis of the Purkinje cells and pyramidal cells in the hippocampus. Mean cell volume and number during ME and MPER showed decrease and increase ($p<0.01$) respectively. It was concluded from the study that mercury exposure impaired memory and motor activities, causes cellular death, loss of neuronal fibers and nuclei fragmentation. Cell size and number were depleted due to mercury toxicity. In MPER period, it restored some cellular morphology, improved behavioral activities, cell size and population.

Keywords: *memory, motor, cell volume, cell number, cellular morphometry*

INTRODUCTION

There is an increase in global risk of exposure to mercury toxicity which poses environmental, human and animal's health in danger. Yet, there are gaps in providing preventive measures to human and animal's health in biota[1]. Several methods were put in place for many decades to control sources of mercury exposure and its release in to the environment. By using scientific understanding of the factors influencing mercury's fate and transport, processes driving mercury production, manifestations and mechanisms of many of its toxic effects in biota[2]. Mercury exists in three forms including: Elemental mercury or metallic mercury which is the element in its pure form. Inorganic mercury compounds or mercury salts, commonly found in nature such as mercury chloride ($HgCl_2$) and Organic Mercury such as methyl mercury etc.[3]Mercury could be found in cosmetics and pharmaceuticals products such as thiomerosal as a preservative in some vaccines[4]. Some medical thermometers used, are filled with mercury although they are gradually disappearing. Among route of mercury exposure is inhalation of mercury vapors, which are monatomic gases that is lipophilic and highly diffusible, given its ability to cross the biological barriers, such as blood brain barrier, placenta, and cell membrane. These can reach the brain or relevant tissues and cause permanent damage[5]. Consumption of contaminated seafood and fishes may contain methyl mercury due to bioaccumulation process. Studies have shown that farm animals can absorb large amounts of mercury in contaminated pastures, which in turn leads to contamination of human

food. Other common exposure pathways include absorption through the skin and breast feeding from women exposed to mercury[6]. Adverse health effects of current Hg levels of exposure vary related to the geographic area[7], with arctic and tropical communities being at high risk of Hg overexposure. Mercury toxicity may aggravate neurological and neurodegenerative[8], metabolic, renal and cardiovascular diseases[9]. According to World Health Organization (WHO) the principal current health concern is effects of mercury on the central nervous system. Listing Hg as one of the top ten chemicals of major public health concern[10,11]. The current studies focused on studying microscopic examination of cerebellar cortex and hippocampal tissues and behavior of rats exposed to mercury toxicity and post exposure recovery.

MATERIALS AND METHODS

Experimental animals: Eighteen Wistar rats weighing averagely 120g were obtained from the department of Human Anatomy, Ahmadu Bello University, (ABU) Nigeria. The animals were allowed to acclimatize for two (2) weeks in the animal house facility of the above department. Animals were fed daily, clean water was provided to feed and drink *ad-bilitum.*

Chemical substance: Mercury chloride was manufactured by May and Baker, Dagenham, England with batch number XN02. The LD_{50} of Mercury chloride was adopted from manufacturer as 166mg/kg body weight. The concentration of 15% and 7.5% equivalent to 12.45mg/kg and 24.9mg/kg of the $LD_{50}$was used in the current study according to Adamu *et al*[12].

Animals Grouping and Hg Administration: Animals were divided into three (3) groups (I, II and III) of six animals per group. Normal saline was administered to group I (control), groups II, and III were administered with doses of 12.45mg/kg and 24.9mg/kg/Hg respectively for twelve consecutive days. After the administration of mercury, each group was further divided into sub-groups “a and b” Groups Ia, IIa and IIIa were sacrifice on the 13th day while groups Ib,IIb and IIIb were allowed for the post exposure recovery period of 12 days without any mercury administration. The rats were fed daily and clean water was provided to feed and drink *ad-bilitum.*

Behavioural Test: Forelimb grip strength test: This test assessed muscle strength and balance[13]. The rats forepaws were placed horizontally on a suspended metal wire 2 mm in diameter, 1m in length and placed 1m above a landing area filled with soft bedding. The length of time each rat was able to stay suspended before falling off the wire was recorded; a maximum of 2 minutes was given to each rat. Each animal was given two trials with a 30 minutes inter-trial rest interval. Forelimb grip strength tests were conducted during both mercury exposure and post-mercury exposure phases.

Short term memory test using novel object recognition: Novel object Recognition test for short term memory in animals was adopted from Ennaceur *et al*[14, 15, 12]. The behavioral protocol include: Habituation, Familiarization and Test phase. In the habituation phase, each animal was allowed to freely explore an open arena in absence of any objects. In the familiarization phase, a single animal was placed in the open field arena containing two identical sample objects (A + A) for about one minute with its back against the object. The animal was then removed and one of the familiar object (FO) was replaced with a Novel object (NO) (A + B). In test phase, the animal was released with its back against the objects and was allowed to explore the objects. Time was recorded for each animal when they explore both FO and NO. The time taken for the animals to explore FO or NO was evaluated as short term memory recognition time. When time is higher in FO or equal to NO then it shows there was memory impairments. The test was conducted for both mercury expose groups and the post exposure recovery groups of animals.

Animal handling: Experimental Animal handling was according to Ahmadu Bello University, research ethics committee (ABUCAMC/HA/0011). Animal were humanely sacrifice a day after mercury chloride exposure i.e 13th day and 25thday of post exposure recovery respectively. Animals from each group were anaesthetize using chloroform. The brain was removed by opening through the sutures of the skull. The brain tissues were fixed in a Bouin's fluid for proper fixation.

H and E Staining Method: The techniques for H and E staining was carried out according to Sheehan and Hrapchak[16]by de-waxing the tissue in two changes of xylene for three (3) minutes each, hydrated by passing them through descending grades of alcohol (100%, 95%, 90%, and 70%) for three minutes each, and then stained in Harris haematoxylin for ten minutes, washed in tap water to remove excess stain. The slides were then flooded with acid alcohol for few seconds for differentiation and then washed in tap water again. The slides were then blued in Scott's tap water for five minutes and counter stained with Eosin for three minutes. The sections were rinsed in tap water, and then dehydrated in ascending grades of alcohol, cleared in xylene. Tissues were then mounted with cover slips using a mounting media. Sections of the tissues were viewed under Leica light microscope and photomicrographs were taken using digital Amscope (MD 900).

Cell Volume and Number: Cellular volume is the amount of space a cell occupies and is found by multiplying the length of the cell by the width and by the height of the cell [17].Neuronal cells are relatively big and generally speaking, the sections examine are thick

enough to show some of the nucleus in each cell. This will be helpful, in counting the nuclei (stained purple with haematoxylin). The cells were counted when nucleus came in to focus in the optical dissector. Digimizer v 4.0 Software was used for calculating the volume and counting cells[18].

Statistical Analysis: Data obtained was expressed as Mean ± SEM (Standard error of mean). One way analysis of variance was use to compare the mean differences between and within the groups. Turkey Posthoc was used for significance, p-value less than 0.05 was considered to be statistically significant. Graph pad v8.0.1 was used for the analysis.

RESULTS

Novel objects recognition test: Grip strength test for motor balance and coordination as shown figure 1a indicates that mercury exposed (ME) groups significantly decreased ($p<0.01$) in time taken during the test period among groups treated with 12.45mg/kg and 24.9 mg/kg when compared with the control group. There was a significant increase ($p<0.05$) in time taken to release the grip strength wire during post exposure recovery among groups treated with 12.45 mg/kg when compared with the 24.45mgkg group. From the short term memory test, there was an increase in the mean latency time taken to explore FO among group treated with 24.9mg/kg which was statistically significant ($p<0.001$) when compared with the control group as shown in Fig1b.In post exposure recovery phase, animal's exploring FO revealed decreases in mean latency time, while time taken to explore NO increases significantly ($p<0.01$) among groups that received low doses of mercuryi.e12.45mg/kg when compare to control and 24.9 mg/kg group as shown in Fig,1b.

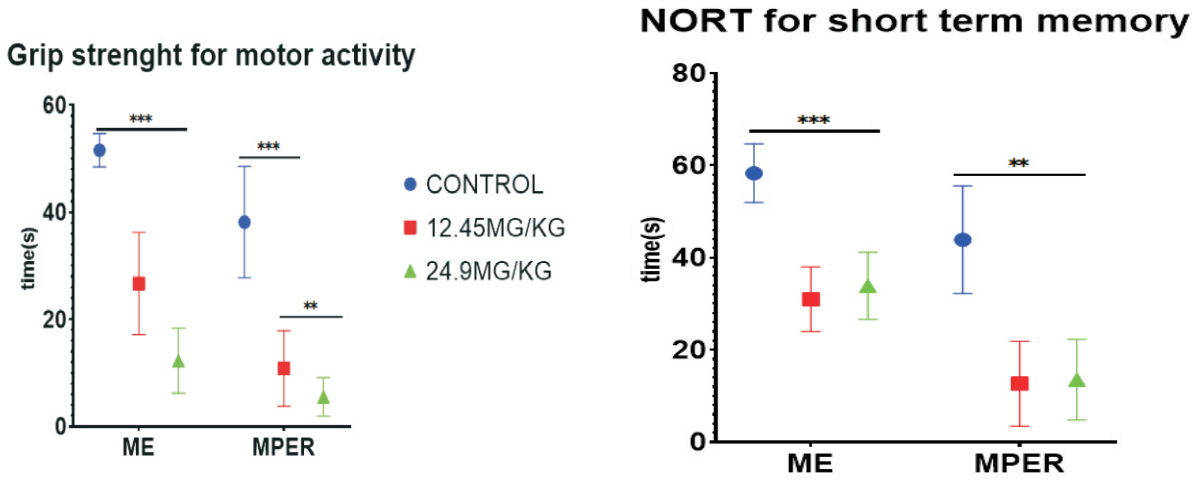

Figure 1: Grip strength test for motor balance and coordination (A). Novel object recognitiontest for Short term memory (B)for mercury exposure (ME) and mercury post exposure recovery (MPER). $p<0.001$***, $p<0.01$**

Histopathological Observations: The micrographs in plate 1 (A-E) reflect CA3 region of the hippocampal tissue with normal cyto-archetecture of pyramidal cell body and some axonal extension in control groups. It was observed among mercury exposed groups B and C (12.45mg/kg and 24.9mgkg respectively) shows distortion in the morphology of pyramidal cell body, condense nucleus or loss of nuclei (karyolysis) with few cells showing nuclei fragmentation (karyorrhexis) as well as scanty population of cells among groups that received 24.9mgkg. Mercury post exposure recovery D and E (12.45mg/kg and 24.9mgkg) groups revealed slight neurodegenerative changes such as karyolysis, pyknotic cells and distortion in the cellular cyto-archetecture of the pyramidal cell. There was slight recovery of the neuronal cells among low dose exposed groups. The results from the histological observations showed normal Purkinje cells, molecular and granular layer with middle Purkinje cell layer as shown in control groups (A). Histological changes in the tissues were observed among groups exposed to mercury doses at 12.45mg/kg and 24.9mgkg (B and C respectively) with evidence of nuclei loss, disintegrated nuclei, and wide area of blue dotted line that indicates loss of Purkinje cell along Purkinje cell layer in groups treated with 24.9mg/kg. During post-exposure recovery phase histological tissues in D and E revealed a normal cyto-archetecture of Purkinje cell bodies (NPc). Some few Purkinje cells still shows disintegrated nuclei or no nuclei.

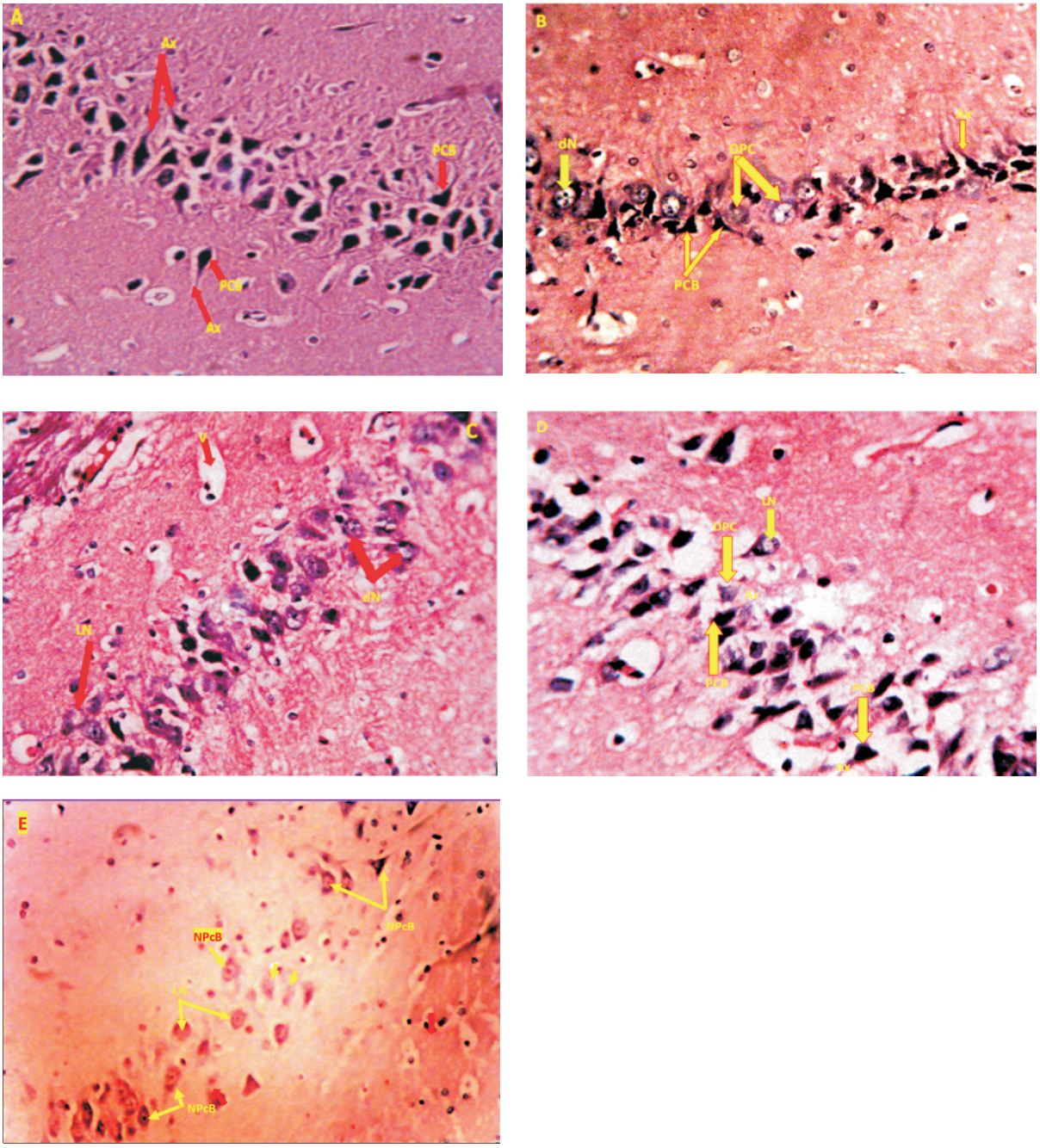

Plate 1: Micrographs A-E are CA3 region of the hippocampal tissue indicating normal pyramidal cell body(PCB), axons (A), degenerating pyramidal cell (DPC), loss of nuclei (LN), disintegrated nuclei,(dN) arrow head pointing at dying cell with total loss of nuclei. Normal pyramidal cell bodies during recovery (NPcB).A is control, B and C are mercury exposed groups (12.45mg/kg and 34.9mgkg. D and E mercury post exposure recovery. H an E, x250

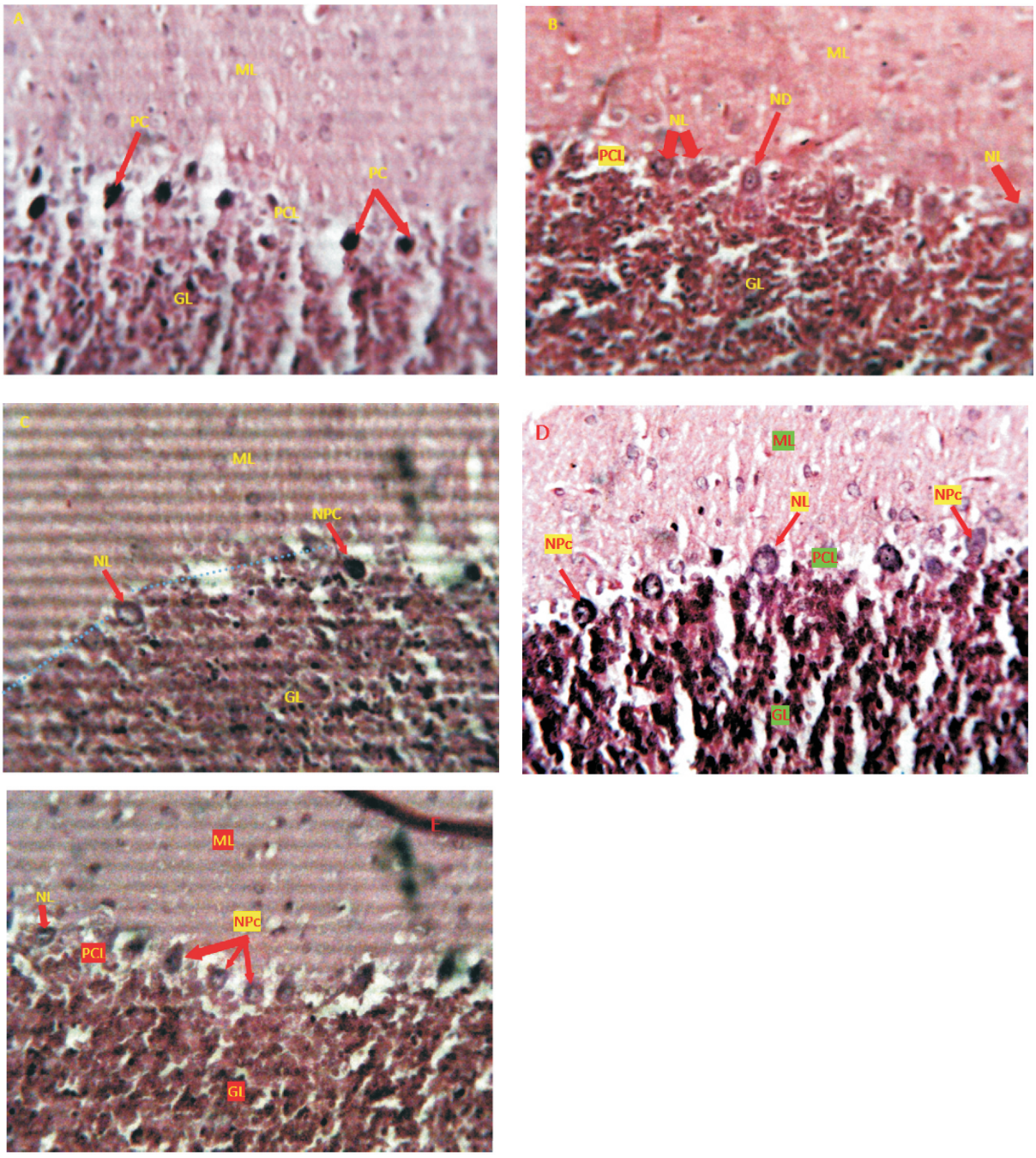

Plate II: Micrographs A-E is a cross section of the cerebellar cortex tissue with normal Purkinje cells (PC), molecular (ML) and granular layer (GL) and middle Purkinje cell layer (PCL), as shown in control groups. (A). Loss of nuclei (NL), disintegrated nuclei, (ND) blue dotted line indicates loss of Purkinje cell along Purkinje cell layer in B and C mercury exposed groups (12.45mg/kg and 24.9mgkg).Normal cyto-archetecture of Purkinje cell bodies (NPc) in mercury recovery phase D and E mercury post exposure recovery. H an E, x250

Mean cellular volume: The mean cellular volume was observed across and within groups for hippocampal and cerebellar volume. The hippocampal volume revealed a decreased that was statistically significant (p<0.01) when compared to the control group during mercury exposure for animals that received 12.45mg/kg and 24.90mg/kg as shown in table 1. Similar observation was made among groups that received 12.45mg/kg and 24.90mg/kg increased significantly (P<0.01) during post exposure period. The mean cellular volume in the cerebellar cortex was observed on Purkinje cells only. Which revealed a significant increase in cellular volume that was higher during post exposure period of mercury among animal that received 12.45mg/kg and 24.90mg/kg to be statistically significant (P<0.01). It was noticed that there was decrease in mean cell volume among animals treated with mercury during mercury exposure period which was statistically significant (p<0.05) when compare to the control as shown in table 1

Table 1: Cytometric analysis of the cell volume and cell count in rats exposed to mercury and post exposure

	Mean Cell volume(nm^3)		
	Control Mean ± SEM(s)	12.45mg/kg Mean ± SEMs)	24.90mg/kg Mean ± SEM(s)
Hippocampus			
Mercury exposure	52.86 ± 9.08	24.59 ± 4.18*	13.99±0.57*
Post exposure recovery	53.66 ± 3.60	50.35± 2.80	21.18 ± 2.00*
cerebellum			
Mercury exposure	23.73±2.83	20.11±1.21*	16.13±1.88**
Post exposure recovery	24.98±6.26	33.70±4.90*	23.75±3.50*

P<0.05; P<0.01** s= time in seconds

Cell count: There was a significant decreased in Purkinje cell number among group thatreceived24.90 mg/kg of Hg when compared to the control and 12.45mgkg groups during mercury exposure phase. Contrary to cell count that was observed among animals in the mercury post exposure recovery phase, treated with 12.45 mg/kg of Hg that was significant (p<0.01) higher in cell number in figure 2A.Pyramidal Cells in the hippocampal tissue decreased significantly (p<0.01) when compared to the control and 12. 45 mg/kg groups. The mercury post exposure recovery period showed an increased that was significantly (p<0.01) higher in animals treated with 24.90mg/kg when compared to similar groups in mercury exposed groups as shown in figure 2B.

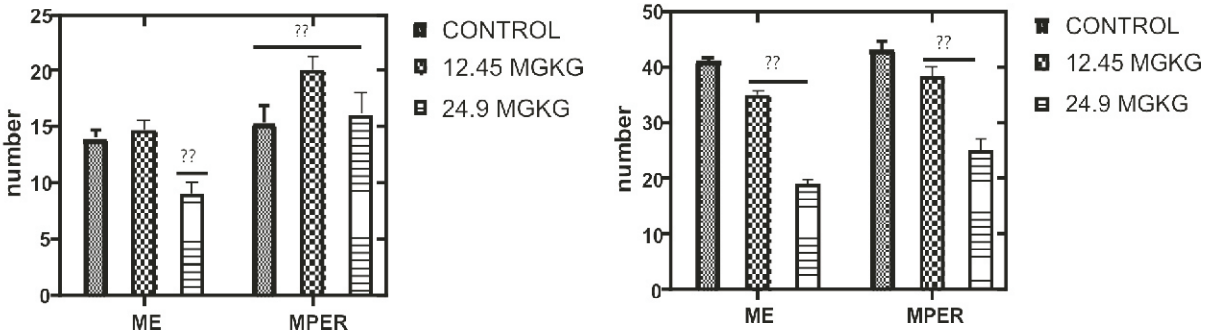

Figure 2: Purkinje cell number count in cerebellar cortex (**A**). Pyramidal cell number count in hippocampal tissue **(B)** for mercury exposure and mercury post exposure recovery. p<0.01**

DISCUSSION

The grip strength test in this study revealed shorter time and quick release of the grip wire during the mercury exposure. It implies that the animals loss motor exploratory skills as well as loss of muscle strength which may be due to the reduction in the functional efficiency of the cerebellum. Pereiraa *et al.,* [19]reported a decreased in swimming performance of fish expose to mercury with reduced time in swimming speed which is similar to the findings of the current studies. During the post-exposure to mercury there was an increase in time taken to release the grip strength wire. This concur to the findings of Pereiraa *et al.,*[19]whom explain improvement of the total swimming distance by *D. sargus* exposed to dietary MeHg for two weeks was resultant of the neuronal cells recovery in medial pallium and optic tectum, and thus related to mechanism associated with motor structural functions.

Decrease in mean latency time taken to explore the novel object among animals administered with mercury chloride indicate cognitive impairment. Previous studies verified that inorganic Hg promoted emotive mnemonic alterations, object recognition and memory and learning[20]. Our study suggest that mercurial exposure causes changes in the AChE transport in the hippocampus that is known to modulate memory, this could be related to a phenomenon of mercury toxicity in the hippocampus which might led to reduced exploratory time during the novel object test in the present studies. Studies of Teixeira et *al*.,[21] also support this argument in relation to glutamates phenomenon. During the post exposure recovery phase, it showed that the mean exploration time among all mercury exposed groups increases when compared to the control group.This implies that there was little recovery of neuronal cells of the hippocampus which lead to higher score in animals. This is in line with Ming and Song[22] who reported that adult neurogenesis might plays a significant role in restoring cognitive functions.

The cerebellum manifested histopathological changes such as loss Purkinje cell layer, apoptosis and necrosis of Purkinje cells. This finding agrees with the report that many heavy metals such as: mercury, lead, cadmium and other organic compounds have the capacity to damage the nervous system [12]. Olga *et al.,*[23] report that Hg-thiol may contribute to apoptosis modulation by interfering with Akt/CREB, Keap1/Nrf2, NF-jB, and mitochondrial pathways. The present study showed that the most affected cells of the cerebellar cortex are the Purkinje cells, this agrees with Ibegbu *et al*., [24] who reported that heavy metals are the most sensitive elements to the cerebellar cortex especially the Purkinje cells which reacts to these noxious substances due to the fact that Purkinje cells are the focal neuron of the cerebellar cortex since all afferent pathways ultimately converge on it and its axon constitute the major exit from the cortex; hence death of the neurons will affect movement, posture and balance.

Increased movement, forelimb strength, geotaxis and rearing behavior were suggested to be due to muscle strength which may mimic recovery from mercury toxicity during post exposure period, this could led to functional efficiency of the cerebellum. Pereiraa *et al.,* [19] reported improvement of swimming activities by *D. sargus* exposed to methyl mercury.

It is known that histopathological changes such as shrinkage of neurons containing pyknotic nuclei in hippocampus and in different regions of the hippocampus are suggestive of neurotoxic effect of mercury in the present study. Degenerative changes in the CA3 hippocampal area were observed, this is known in retrieval and consolidation of stored activity. According to Wilson *et al*., [25] reported that lesion of lateral entorhinal cortex and hippocampus can impair object recognition and other memory related activities. Ceccatelli *et al.*[26] suggested that Mercury induces apoptotic cell death via different pathways such as mitochondrial/caspase-dependent pathway; the $Ca^{2+/}$calpain pathway; the involvement of lysosomal enzymes such as cathepsins; the translocation of AIF (apoptosis inducing factor) into the nucleus with subsequent caspase-independent apoptosis can led to cellular death. Post exposure recovery in general could be due to new cells growth in different brain region. Findings concur with the work of Ming and Song, [23] who reported that neurogenesis plays a role in neuronal development. It also agrees with the Zheng *et al.,*[27] who reported that neurogenesis is an important therapeutic target in treating neurological disorders

Olga et al., [23] reported that Mercury-induced inhibition of enzymatic activity and ability to cross the blood barrier are some of the important factors in Hg toxicity which can results to reduced cell size and number. Cytometric evaluation in the present study shows changes in number and volume of cells during mercury exposure. It is important to note that Mercury can also easily permeate the cell membrane and bond to the intracellular free sulfhydryl group of glutathione and proteins; therefore mercury exposure could be linked to cellular redox Imbalance. Moreover, demolishing the cell structure and obstructing nutrient transport. This will aggravate the toxicity to organisms. This is in line with the work of Katherine *et al*., [28]who reported that change in cell volume could impair enzyme balance, substrate concentration and energetic metabolism of the cell which can lead to apoptosis. Studies of Teixeira *et al.,* [21] reported that Hg exposure and neuronal death are related to decrease in number of neuronal cells which can consequently alters neurochemical balance of some neurotransmitters.

CONCLUSION

It was concluded that Mercury exposure impaired motor and cognitive functions in animals models in the present study which consequently relates to histopathological observations such shrinkage, nuclei loss and fragmentation, reduced cell volume and number in the cerebellum and hippocampus. The post exposure recovery from mercury toxicity was proved to be a natural process of neuronal healing after mercury toxicity exposure. Restoration of grip strength, memory activities and increased cellular number were suggestive to be recovery process from mercury toxicity which could be due to neurogenesis and its processes.

Conflict of Interests:

Authors declare no conflict of interest

Acknowledgement

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