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β-Amyloid Accumulation Impaired Short-Term Memory in Mercury Treated Rats

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ABSTRACT

Mercury is a non-essential element that exhibits a high degree of toxicity to humans and animals. The study was designed to assess β-amyloid accumulation and its role on short-term memory impairment in mercury treated rats. Twenty four Wistar rats of average weight 190 g were divided into four groups of six animals per group. Group I served as control, while other groups were administered with mercury chloride orally at 12.45 mg/kg, 24.9 mg/kg and 49.8 mg/kg, i.e. low, medium and high doses respectively, for 28 days. Short-term memory test was assessed using novel object recognition test. Animals were humanely sacrificed; brain tissues were fixed in RCL_2 fixative. The hippocampal tissues were used for histopathological studies using routine haematoxylin and eosin techniques and Congo red stain for the presence β-amyloids. Acetylcholinesterase (AChE) enzyme was determined using AChE assay kit cytometric analysis for cell volume and number was performed using Digimizer v4.0. There was a significant increase ($p < 0.01$) in mean time for animals exploring familial objects among rats that received 24.9 mg/kg (low) and 49.8 mg/kg (high) of mercury chloride. Histopathological observation showed neurodegenerative changes in the hippocampus. Expression and deposits of β-amyloid protein was observed in animals treated with 24.9 and 49.8 mg/kg body weight of mercury chloride. AChE significantly decreased ($p < 0.001$) among groups that received low, medium and high doses of mercury chloride. It is concluded that mercury impaired short-term memory, induced beta amyloid accumulation, alters AChE concentration and also causes histopathological lesion in the hippocampus.

Key words: *Mercury, β-amyloid, Hippocampus, Short-term memory, Cell volume, Cell number*

INTRODUCTION

Mercury has been used worldwide for many centuries for commercial and medicinal purposes (Ibegbu et al. 2014). Man in his environment is exposed to much potential hazards by heavy metals via bioaccumulation and biodegradation which is transferred to man via food chain due to anthropogenic activities (Wang et al. 2007; Chen et al. 2016; Bjørklund et al. 2017). Mercury exists in three forms: These forms include; Elemental mercury also called metallic mercury, this is the element in its pure, un-combined form; Inorganic mercury compounds or mercury salts are more commonly found in nature, which include mercuric sulphide

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(HgS) and mercuric chloride ($HgCl_2$). Organic mercury is formed when mercury combines with carbon and other elements. Examples are dimethyl mercury (Al-saleh et al. 2016; Sari et al. 2016). Some sources of mercury and its compounds include industrial sources which are mercury emitted from fossil fuels and into the air by mining co-operations, water bodies and land (Lim et al. 2010; Bjomberg et al. 2011). There are many routes of exposure to mercuric compounds, but the evidence of exposure is dependent on the level of toxicity (Chen et al. 2016; Ye et al. 2016). These exposure routes include: Oral exposure which can be via consumption of food products and grains preserved with mercuric compounds (WHO 2005). Inhalational exposure route can be from fumes, industrial actions of fossil fuel, odour and sewages in the form of mercuric oxide (Dórea 2015). Dermal exposure can be through the use of mercuric ointments, creams and some soaps which can result in disease conditions (Chan 2011). Mercury and its compounds have been shown to also have effects on growth, weight, renal system, liver, enzymes, memory and psychological disturbances to mention but a few (Valera et al. 2008). Signs and symptoms of mercury poisoning include; irritability, excitability, restlessness of the skin and eyes, headache, dizziness, difficulty in breathing and frequent urination (ATDRS 2011). Mercury has no known nutritional or biomedical importance but has various applications and uses; such as preservation, employed by pharmaceutical company, agriculture and in cosmetic production (WHO 2005). The hippocampus is one of the brain's great mysteries which play a critical role in the formation, organization, and storage of new memories, as well as connecting certain sensations and emotions to these memories. One of the memory types is short-term memory which involves the hippocampus, and allows recall for a period of several seconds to a minute without rehearsal. Its capacity is also very limited (Jeneson and Squire 2012). The study aimed at evaluating whether exposure to inorganic mercury is capable of generating cognitive alterations and/or biochemical modulation, tissue damage and cell death in the hippocampus. The study was designed to evaluates the effect of mercury on β-amyloid accumulation in the hippocampus and short-term memory impairment in mercury treated rats.

MATERIALS AND METHODS

Experimental Design

Twenty four male adult Wistar rats were obtained from the Animal's House of the Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria. Nigeria. The animals whose body weights were between 160-190 g, were housed in polyester cages with wire gauze covering. The animals were allowed to acclimatize for two weeks in the animal house of the Department of Human Anatomy, Ahmadu Bello University, Zaria. Nigeria. Animals were fed with grower's mash brand of the animal feed, while clean water was provided in plastic drinking bottles, and rats were allowed to feed and drink *ad libitum*. Animals were randomly divided into four groups with six animals per group. And the administration of the mercuric chloride lasted for twenty eight consecutive days, which was done orally using a syringe. Experimental animal handling was carried out according to Ahmadu Bello University, Zaria Research Ethics Committee: ABUCAMC, 2016.

Mercuric chloride used in the present study was manufactured by May and Bakers Limited, Dagenham England (XN202). Acetylcholinesterase Colorimetric assay kit (ab138871) was purchased from ABCAM PLC United Kingdom.

Experimental Protocol

The LD_{50} of mercuric chloride was adopted from ATDRS (2011) as 166 mg/kg body weight. The doses of mercury chloride used was determined using 7.5%, 15% and 30% of the standard LD50 per kilogram body weight according to the methods of Ibegbu et al. (2014). Animals in Group I served as control and were given 2 mL normal saline, while animals in groups 2, 3 and 4 were administered low, medium and high doses of mercuric chloride at 12.45, 24.9 and 49.8 mg/kg body weight respectively.

Table 1: Effects of Mercury on Exploratory Mean Latency Time Taken for Short Term Memory Test using Object Recognition Test

Weeks	Test	N	Control	12.45 mg/kg	24.9 mg/kg	49.8 mg/kg
			Mean ± SEM			
Week 1	FO	6	3.00±0.25	2.17±0.41	5.33±1.45	3.00±0.57
	NO	6	6.17±0.98	3.00±0.45	3.17±0.87	1.50±0.22
Week 2	FO	6	8.50±1.94	11.07±1.94	10.00±2.05	9.67±2.65*
	NO	6	7.00±1.70	8.70±1.30	6.17±1.85	4.83±1.75
Week 3	FO	6	6.17±1.68	9.50±2.65	34.33±8.10*	36.00±5.66*
	NO	6	6.00±1.53	8.83±2.56	10.17±2.10	12.67±2.81
Week 4	FO	6	8.29±1.60	13.71±2.96	38.43±13.10*	52.57±17.47**
	NO	6	25.60±4.55	11.60±1.63	16.40±3.14*	14.00±6.34

* $p \leq 0.05$; ** $p \leq 0.01$; n = number of animals per group; s= time in second; FO= Familiar Object; NO= Novel Object

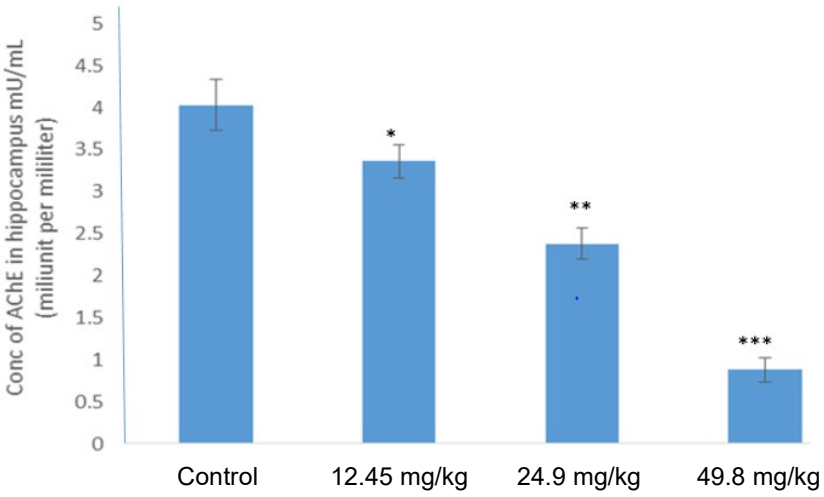

Figure 1: Concentration of AChE in the Hippocampus of Rats Treated with Mercury. * p < 0.05; ** p < 0.01; *** p < 0.001

Behavioural Test

Novel Object Recognition Test for Short-Term Memory

Novel object recognition is a highly validated test for recognition memory. It can be used to test the efficacy of memory enhancing compounds such as the effects of certain compounds on memory and the influence of genetics or age on memory (Goursaud et al. 2015; Richler et al. 2017). The rats were exposed to two similar objects to explore for a minute in order to get familiar with the objects as familial objects (FO). Then one of the familial objects was replaced by another novel object (NO). The time taken to explore the FO and NO was recorded as mean exploratory time (in seconds) for objects recognition according to the method of Clipperton-Allen and Page (2014). The experiment was repeated weekly for assessment of short-term memory. If memory is functioning normally, the rats spend more time exploring NO than it does exploring the FO. But when exploration of NO and FO is the same, or higher in FO, this can be interpreted as a short-term memory deficit (Clipperton-Allen and Page 2014).

Termination of Experiment

Animals were humanely sacrificed a day after the last administration of mercuric chloride (29th day). The animals were anesthetized using chloroform by inhalation and the brain tissues were removed by opening through the sutures of the skull using a brain dissector. The brain tissues were then transferred into specimen bottles containing RCL_2 fixatives and fixed for 24hrs according to Moelans et al. (2011) for histopathological examinations.

Tissue Processing

Brain (hippocampus) tissues were processed routinely for histopathological studies using haematoxylin and eosin staining procedure and Congo red staining techniques for β-amyloid accumulation. The fixed hippocampal tissue were removed from the RCL_2 fixative and dehydrated using ascending grades of alcohol. Dehydrated tissues were then cleared in two changes of xylene for two hours, and then infiltrated by immersing in molten paraffin wax and allowed to solidify. The embedded tissues were blocked in a rectangular block. Rotary microtome was used in cutting the tissue sagitally at 10 µm per section. Tissues were allowed to float in a warm water bath of about 30ºC to help in spreading the paraffin ribbon, clean glass slides were used to pick up the tissue from the water bath.

Hematoxylin and Eosin (H and E) Staining Techniques

Hippocampal tissues were allowed to dry by dewaxing the tissues in two changes of xylene for 3 minutes each. Descending grades of alcohol; 100%, 95%, 90% and 70% was used to hydrate the tissues for 3 minutes each, stained with Harris haematoxylin for 10 minutes, and washed with tap water to remove excess stain. The tissue slides were flooded with acid alcohol for some seconds for differentiatio- n, washed in tap water again. Bluing was done in water, differentiated in 70% ethanol and then stained with eosin. Section were washed and used for histological observation.

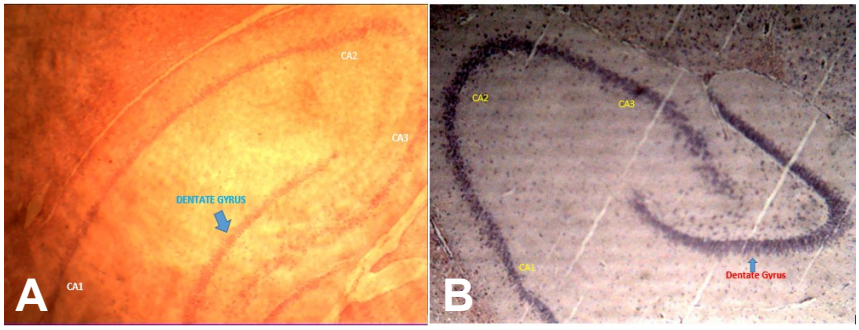

Figure 2: Sections of the hippocampus stained with H and E (A) showing CA1, CA2, CA3 and dentate gyrus. B, Section of the hippocampus stained with Congo red showing CA1, CA2, CA3 area and dentate gyrus. × 40.

Congo Red Staining Technique

Sections of the hippocampus were deparaffinized, wash with water and stained with Congo red solution for 1 hour, then rinsed in distilled water and differentiated quickly in alkaline alcohol solution. The tissues were rinsed in tap water for 5 minutes and counter-stained in haematoxylin for 5 minutes. They were rinsed in tap water for 10 minutes, and dehydrated through changes of 95% and 100% alcohol, cleared in xylene. This was followed by mounting on glass slides for viewing under light microscope MD900 Am scope microscope digital camera which demonstrate-d the amyloid as red and nuclei as blue. Tissue slides were viewed under light microscope (Leica Microsy-stem Inc, Tokyo, Japan) and photomicrographs were made using digital Amscope (MD-900) microscope camera.

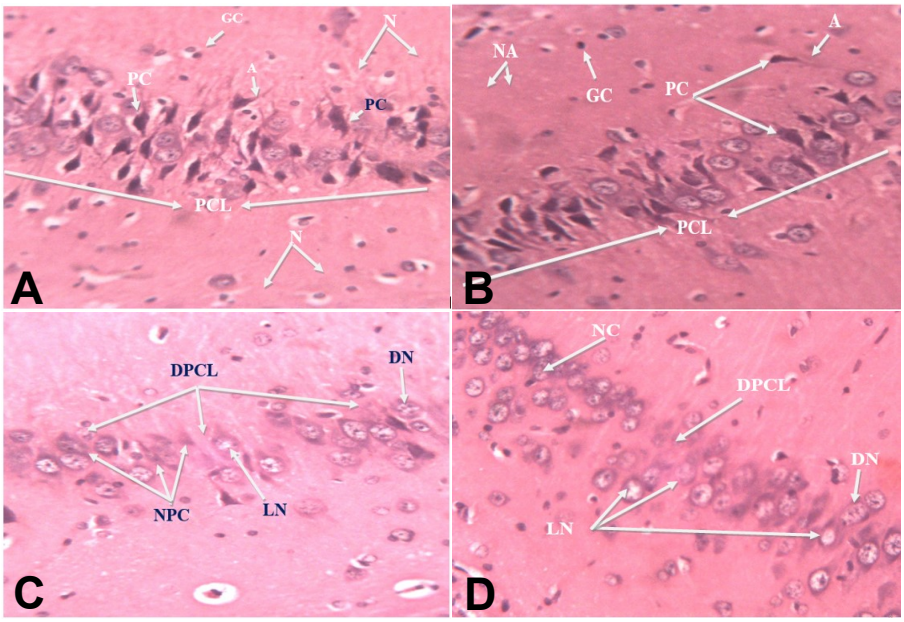

Figure 3: Sections of the hippocampus (CA1 region). A Control (I) showing normal pyramidal cell layer (PCL) with normal pyramidal cells (PC), glial cells (GC) and Neuropil (N) area B. low dose (12.45 mg/kg), showing normal pyramidal cell layer (PCL) with normal pyramidal cells (PC), Axon (A). Glial cells (GC) and neuropil (NA) area of the CA1 region of the hippocampus. C medium dose degenerated pyramidal cell layer (DPCL) with disintegrated pyramidal cells nuclei (DN), loss of nuclei (LN) and Necrotic cell (NC) D. high dose (49.8 mg/kg), showing distorted pyramidal cell layer (DPCL) with disintegrated pyramidal cells nuclei (DN), loss of nuclei (LN) and Necrotic pyramidal cell (NPC). CA1 region of the hippocampus (H&E ×250)

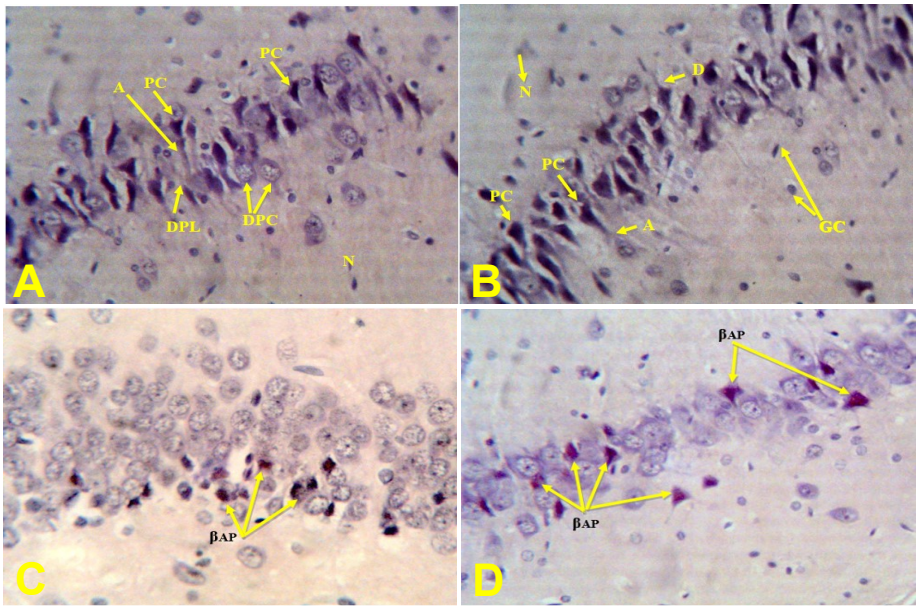

Figure 4: Sections of the hippocampus (CA1 region). Control (I) with normal pyramidal cells (PC) glial cells (GC) axon (A), dendrites (D) and neuropil (N) area F low dose (12.45 mg/kg), showing normal pyramidal cells (PC), degenerating pyramidal cells (DPC), area of degenerated pyramidal layer (DPL) Axon (A) and Neuropil (N) area G medium (24.9 mg/kg), showing accumulated beta amyloids proteins (βAP) H. high doses (49.8 mg/kg) of mercury showing degenerated pyramidal cell layer with loss of neuronal fibers and accumulated beta amyloids proteins (βAP) accumulation (Congo red ×250).

Neurochemical Analysis (Acetylcholinesterase Assay)

Hippocampal tissues were collected and prepared according to the method described by Zatta et al. (2002). The tissues were placed on an inverted petri dish on ice. and homogenized in 10 mL of a medium containing a solution of 0.1 M sodium phosphate 10 w/v (pH 7.4). The total homogenate was centrifuged at 1000 rev/min for 7 minute. The supernatants were used for AChE enzyme activity determination according to manufacturer's instruction; AChE assay kit (Colorimetric) ab138871 (Abcam, 2016)

Statistical Analysis

Data obtained were expressed as Mean ± SEM (standard error of mean). One Way Analysis of Variance was employed to compare the Mean difference between and within

the groups. Cytometric analysis was performed using Digitizer v4.0. P-Value less than 0.05 was considered statistically significant. Statistical analysis was performed using SPSS-IBM,v20. Chart were produced using Microsoft(R) Excel 2007 for windows..

RESULTS

Evaluations on Effects of Mercury on Short-Term Memory using Object Recognition Memory Test

The results showed that there was a decrease in the mean time taken for the animals to explore the novel object in the Control group, though the decrease was not significant as shown in Table 1. The results showed that animals that received 12.45 mg/kg had an increased in the mean latency in exploring the novel object (NO) though the increase between weeks 1, 2 and week 3 was not significant, but significant increase was observed in week 4 for animals exploring the familial object (FO). There was an increased in the exploratory time in NO which was not significant, but significant increase in the meantime was observed for animals exploring the FO between weeks 2 and 3, between weeks 3 and 4, and between weeks 1, 2, 3 and 4 respectively in both Groups of animals that received medium and high doses of mercury (24.9 mg/kg and 49.8 mg/kg respectively) as shown in Table 1.

Effects of Mercury on AchE Concentration

There was a significant decrease ($p < 0.001$) between control group, 12.45 mg/kg, 24.9 mg/kg and 49.8 mg/kg in concentration of AChE. A decrease in concentration of AChE groups that received 24.9 mg/kg was significant (***P<0.01) when compare with control and groups that received 12.45 mg/kg of mercury. Animals treated with 12.45 mg/kg show a decrease which was significant (*P<0.05) to groups treated with of 24.9 mg/kg and 49.8 mg/kg mercury as shown in Figure 1.

Histopathological Observation of the Hippocampus

The histological examination of the hippocampus stained with H and E (A) with CA1, CA2, CA3 and dentate gyrus. While B, is a cross-section of the hippocampus stained with Congo red showing CA1, CA2, CA3 area and dentate gyrus as shown in Figure 2.

Figure 3 showed a cross section of animal's tissue in the Control (I) group had normal histological features, pyramidal cell layer, pyramidal cells, glial cells and neuropil area of the CA1 region of the hippocampus as shown in Figure 3A. Animals in group II that received low (12.45 mg/kg) does not showed any vivid histopathological changes in Figure 3B. Section of the hippocampus of animals in group 3 that received medium (24.9 mg/kg) dose of mercury chloride showed distorted pyramidal cell layer, disintegrated pyramidal cells nuclei, loss of nuclei and necrotic pyramidal cell in CA1 region of the hippocampus as shown in Figure 3C. Animals in group 4 administered with high doses (49.8 mg/kg) showed distorted pyramidal cell layer with disintegrated pyramidal cell nuclei, loss of nuclei and necrotic pyramidal cells in CA1 region of the hippocampus as shown in figure 3D. The Congo red staining techniques did not express beta amyloids proteins in control (I) and animals given low dose of (12.45 mg/kg) mercury as shown in Figure 4E and 4F. Animals that received medium and high doses, i.e. 24.9 mg/kg and 49.8 mg/kg of mercury showed beta amyloid protein deposit in the CAI region of the hippocampus as shown in Figure 4G and 4H.

Cytometric Analysis

Table 2 indicates a significant decrease ($p \leq 0.05$; $p \leq 0.01$) in cell volume among groups of rats administered with 12.45 mg/kg ($p \leq 0.05$) when compare with the control.; 24.9 mg/kg ($p \leq 0.05$) and 49.8 mg/kg ($p \leq 0.01$) shows a decreased cell volume as compared with control and groups that received medium and low doses at the CA1 region of the hippocampus. Animals among Groups treated with 24.9 mg/kg when compare with the control and low dose group (12.45 mg/kg) shows a significant ($p \leq 0.01$) decrease while 49.8 mg/kg of mercuric chloride showed a significant decrease ($p \leq 0.01$) between low (12.45 mg/kg), medium (24.9 mg/kg) and control group in cell number.

Table 2: Cytometric Analysis of Temporal Lobe of the Cerebrum in Adult Wistar Rats Orally Exposed to Mercuric Chloride

Parameter	Control	12.45 mg/kg of mercuric chloride	24.9 mg/kg of mercuric chloride	49.8 mg/kg of mercuric chloride
Cell volume (nm^3)	84.30±13.76	66.47±1.76*	36.38±2.28*	25.63±6.74**
Cell number	34.00±3.77	33.50±2.53	27.33±2.60*	14.00±2.88*

nm^3 (nanometer cube); $p \leq 0.01$**; $p \leq 0.05$*

DISCUSSION

Short-term memory deficits in the present study was attributed to degenerative changes observed in the hippocampus and possible alteration in the level of AchE. The hippocampus plays a crucial

role within the nervous systems for long term memory, but little if any role in the short-term memory retention (van der Schaaf et al. 2013). The increase in mean latency taken by the experimental rats to explore familial object during short-term memory recognition could be dose dependent which was attributed to decrease in cell volume and number. Conversely, the pyramidal cell layer of the hippocampus appears to be damaged with degenerated cells, vacuolated spaces and distortion in the general morphology of the pyramidal cells which appeared smaller than normal. These alterations in the hippocampus was due to mercury exposure which could consequently, result to impaired memory in the present study. Findings from the report of Albores-Garcia et al. (2016) showed that recognition index indicated by mercury exposure impaired recognition memory in a dose-dependent manner in animals. Degenerative changes observed in this study, can hinder the hippocampus from detecting or extracting significant information for further memory consolidation and from repetitive activity that was already learned or remembered.

Acetylcholinesterase enzyme (AChE) is widely distributed in the central and peripheral nervous systems (PNS), and the motor end-plates of the skeletal muscles and electric organs (Rajathi and Selvi 2011; Morissette et al. 2016). It was observed in the present study that, there was a significant decrease in AChE concentration in the cerebrum of animal treated with 48.45 mg/kg of inorganic mercury. This implies that depletion of AChE concentration in the brain can alter cognitive efficiency, memory consolidation and retrieval of information (Morissette et al. 2016). Short-term memory impairment observed in the present study could also be associated with AChE inbalance. Stamler et al. (2016), reported that depletion of acetylcholine (ACh) and decreased cholinergic activity, predominantly in the neocortex and hippocampus, are associated with cognitive decline in Alzheimer Disease. Dinesh and Kapil (2016) and Yanjing et al. (2016) had reported impaired spatial and non-spatial learning and memory abilities which could be, at least partially, due to the decreasing activity of AChE in aged control mice exposed to mercury.

The hippocampus is a structure related to memory and learning (Jingwei et al. 2016). Result from the present study revealed that the pyramidal cells of the hippocampus showed some changes such as degeneration and reduction in the number of pyramidal cells, loss of neuronal cell fibres, reduced number of cell sizes when compared to the Control Group. This could be as a result of the exposure to mercury chloride. These changes imply that the activity of the hippocampus in memory formation and learning will be impaired and the role of the hippocampus that involved storage and retrieval of information will also be lost. Findings from the present study agree with the results of Jingwei et al. (2016) which showed that exposure to mercury caused changes in the ultrastructure of the neurons and morphological changes in the hippocampus, resulting significant damages.

Extracellular accumulation of amyloid beta protein (Aβ) plays a central role in Alzheimer's disease (AD) (Kin et al. 2014). Some metals, such as copper, lead, and aluminum can affect the Aβ accumulation in the brain. However, the effect of mercury on Aβ accumulation in the brain is not clear (Ji-Won and Byung-Sun 2013). Thus, the current study demonstrated that mercury induces Aβ accumulation in the hippocampus. According to a recent study, mercury exposure contribute to Alzheimer's disease in animals or man (Ji-Won and Byung-Sun 2013). This is in contrast to Dong et al. (2014) who reported that mercury pathogenesis of AD is not completely understood. According to an *in vitro* study by Per and Lennart (2012) mercury increased the secretion of Aβ. However, this result was not confirmed in an in vivo study by Olivieri et al. (2000). Moreover, the present study investigated only the effect of mercury on Aβ levels and provided few explanations on the mechanism of beta amyloid accumulation due to mercury intoxication. Expression of Aβ level in hippocampus in the current study was found to be dose dependent. High and medium doses of exposure expressed more Aβ accumulation in the hippocampus. The findings from Maqbool et al. (2016), showed that increased Aβ levels in the medium and high doses exposure to mercury were in a dose and time dependent manner and as such mercury is considered as one of the potential exogenous factors responsible for AD pathogenesis according to Alzheimer's disease International (ADI 2013).

The deposits of Aβ in the hippocampus in the present study could interfere with neuron-to-neuron communication at synapses and contribute to cell death, which could be the possible explanation for memory impairment in Wistar rats in the present study. Decreased cell volume of the hippocampal cells in the current study can also hinder normal functions of the hippocampus to detect or extract significant information for further memory consolidation and from repetitive activity that was already learned or remembered to be lost (Šimić et al. 2016). Simi et al. (2016) also reported that, Aβ was toxic to neurons. In the brain, Aβ causes loss of long term potentiation, damages synapses and kills neurons (Sherin and Sumathi 2016). Moreover, mercury showed selective neurotoxicity for the hippocampus and entorhinal cortex (areas that are severely affected in AD). While results from the study of Wise (2016) did not support these findings, but report provided reassurance that, exposure to mercury, especially via seafood contaminated with mercury was not related to increased Aβ level or any brain pathology.

Decrease in cell volume and number in the present study implies that intracellular biochemical reactions such as enzymes balance and substrate concentration and energetic metabolism could be impaired which can trigger programmed cell death. This finding agrees with Allen et al. (2016) who reported that Cell volume changes induced by HgCl provide further evidence for a primary membrane effect of mercury. Mercury not only increased or decreased cell volume, but also prevented the normal volume regulatory decrease or increase after the swollen of cell in hypotonic media which could led cell death. Ige (2012) reported that Na/K–ATPase is more sulfhydryl protein that is sensitive to mercury and as such mercury can inhibits 86Rb transport mediators for Na /K transport and alter cellular structure.

Conclusion

Short-term memory impairments was attributed to be dose and time dependent due to mercuric chloride intoxication. Histopathological observations, such as pyknosis, loss of neuronal fibers, alteration in AchE level, decreases in cell numbers and cell volumes, congested cytoplasm and eventually cell death in the hippocampus was also observed. Accumulation of beta amyloid proteins in the hippocampus was shown as histopathological marker that could impair memory.

Conflict of Interest

None declared.

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