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Immunohistochemical Investigation of *the Activities of Neuron-Specific-Enolase and Motor Coordination* in the Cerebellar Cortex of Lead-nitrate Treated Rats.

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ABSTRACT

Damage to the cellular components of the cerebellum, altered motor coordination and ataxia has not been reported as findings in studies addressing lead-induced toxicity in experimental studies. This study aimed at finding out the effects of lead exposure on the cellular profile of the cerebellum and also tests for motor coordination in juvenile male rats. 20 juvenile male rats were randomly assigned into control (A) and lead-exposed (B) groups. The rats in group A were exposed to normal saline, while rats in group B were exposed to 100 mg/kg body weight of lead-nitrate. The duration of exposure was for 30 days. At termination of administration, motor coordination in the lead-induced group was significantly altered when compared with control group, while the histological section showed marked distortion with evidence of necrosis in the morphology of the Purkinje cells in the cerebral profile of the rats in the lead-induced group. The neuronal cells in the cerebral profile of the rats in the control group was well preserved. There was no marked changes in the motor coordination of the rats in the control group.It was observed that exposure to lead conferred neurodegenerative effects on the Purkinje cells in the cerebellum and also alter motor coordination.

Key words: - brain, environment, excavation and mining, excitotoxin, heavy metal, neurons.

INTRODUCTION

Lead (Pb) is a non-physiological metal with a relative atomic mass of 207.2. It has been used by humans from time immemorial. The southeastern Europe utilizes it as wine sweetener, and in more recent times, it is used as additives in the production of gasoline, jewelries and ornaments, ammunitions and bullets, and also in the fabrication of alloys [1,2,3,4].

Unknowingly, a large number of the residents of industrialized cities are exposed to environmental Pb levels, thereby affecting many organs and systems in the body and this could have a corresponding deleterious effects on their health[5,6,7]. Because of its high degree of toxicity, intoxication from Pb is usually determined by a variety of gastrointestinal, hematological, neurological, and renal symptoms [8].

The studies of Xu *et al.*[9] and Flora[10] have shown that Pb alter neurotransmitters levels, increase reactive oxygen species (ROS), induce DNA damage, induce apoptotic characters on cells (through the release of cytochrome *c*), and also impair the functional integrity of mitochondria.

The exposure of human to Pb may occur through ingestion, inhalation, and through the skin. Pb may be ingested directly from contaminated air, soil, and water, and indirectly by oral consumption of contaminated nutrition (such as animal meat, fruit and vegetables and their derivates).

Environmental, industrial and/or occupational Pb exposure may be aggravated by poor protective behaviours, poor hand-to-mouth habits, and socio-economic factors. Lead is found in food, batteries, solders, plastics, household paints and gas, but also in glass feeding/nursing bottles, toys, earthen pots, herbal remedy, wild game, jewelries and cosmetics. Depending on the degree of poisoning (which also correspond to the level of Pb in the blood), Pb is capable of causing anemia, encephalopathy, hypertension, nephropathies, and sterility.

Some recent studies have discovered other significant health effects of lead exposure, these include hypertension and other cardiovascular consequences [11] and renal disease [12]. Chronic lead exposure in adults can also result in cataracts, impaired memory, infertility,

joint and muscle pain, and nerve disorders. Intense lead exposure can cause diverse of neurologic disorders, such as convulsions, coma, and impaired muscular co-ordination.

In vivo studies [13,14,15,16] of animal models of lead-induced neurotoxicity have centered on indirect evidence for neuronal cell death after lead exposure. Although in vivo evidence of neuronal cell death in rat brain has been described, degeneration of specific neurons observed in multiple brain regions, and the manner in which they undergo degeneration, has only recently been identified [17].

Several mechanisms of lead neurotoxicity have been proposed. These include vasculopathy resulting in breakdown of the blood-brain-barrier, intracranial hemorrhage and/or cerebral edema; modification of synaptogenesis; disruptions of energy metabolism, calcium homeostasis, and cell signaling; alterations in neurotransmitter systems, particularly the glutaminegic system and its effects at N-methly-D-aspartate glutamate receptors [18,19].

According to Adewole and Ayoka [20], lead-induced impairment occurs preferentially in the prefrontal region of the cerebral cortex, cerebellum, and hippocampus. Lead toxicity may damage the basal forebrain and the primary visual cortex, and cause changes in the permeability of capillaries in the cerebral cortex [21].

The cerebellum is the largest neural structure of the central nervous system implicated in the control of voluntary movements. Almost all information derived from ongoing motor commands issued by cortical and subcortical circuits is accessible by the cerebellum, which in turn indirectly projects to and modulates the activity of motor neurons that drive the muscles. Cerebellar lesions lead to a loss of movement precision and postural instability, interpreted by classical terms such as ataxia, tremor, and dysdiadochokinesia, and on the other hand, they impair motor learning. The most immediate function of the cerebellum is therefore to monitor the progress of each individual movement and ensure that the desired goal is accurately reached by tweaking, as necessary, the underlying cortical, brainstem, or spinal motor pathways.

The concentration of neuron specific enolase (NSE) is high in the neuronal cells present in the central nervous system. The immunohistochemical demonstration of *NSE* is specific as anti-*NSE* antibodies stained neurons are localized only in the nervous system [22]. The *NSE* is an enzyme of the glycolytic pathway that catalyzes the conversion of phosphoglyceric acid into phosphopyruvic acid [23]. During exposure to lead (Pb), the neurons of the CNS respond to the toxic assault differently due to the different neurotoxic pathways established in many regions of the CNS.

A major process occurring during several form of neuronal toxicity is the disruption of glucose metabolism [24]. *NSE*, falls into the category of acid soluble enzyme required in the pathway that metabolizes glucose [23,24]. It is an effective indicator of the shift in metabolic activity in neuronal cells [25]. A neuron will undergo certain alternation of morphological sequences following heavy metal exposure. Zhou *et al.,*[25]suggested that under the influence of neurotoxin, the levels of *NSE* activity in the neurons is altered in a way that it changes the ontogenesis levels thus modifying the level of this enzyme above the regular required level. It is also possible that this phenomenon will occur during *DNA* fragmentation to augment synthesis of nuclear material thereby leading to repair of the genetic material as a standard response of the cell to survival by developing adequate and sufficient ATP for maintaining the neurons [26].

Taking into consideration the reported effect(s) of lead-exposure on various organs of the living body most especially the brain, the aim of this study is to observe the level of energy utilization using *NSE* activities changes within the neurons of the cerebellum of juvenile male rats in the control and $PbNO_3$-treated groups respectively, and to associate these changes, if any, with motor coordination by using the modified rotarod motor coordination test.

MATERIALS AND METHODS

Animal Care and Treatment

All animal care procedures were conducted in accordance with guidelines developed by the National Academy of Science and approved by the Ethical Committee on research of the Institute of Public Health, College of Medicine, Obafemi Awolowo University, Ile-Ife, Nigeria. This study included 20 juvenile male rats (Wistar strain) with body weights ranging from 45 to 60 g.The rats were housed in two separate polycarbonate cages with stainless steel wire lids. Two treatment groups were established:

group A (control); were orally dosed with normal saline (NS) alone on a daily basis for 30d

group B; treated orally with 100 mg/kg lead-nitrate ($PbNO_3$) for 30d.

Motor Coordination Activities

Motor coordination activities in the experimental rats was examined by utilizing a rotarod treadmill. The motor coordination activities was examined in the experimental rats once every five days within a thirty day period. Five rats from each of the experimental groups were observed on this device for 15 minutes within an hour period. Each of the test rats (irrespective of the group) were allowed to walk for 2 minutes on the treadmill. Any of the rats that walked for 2 minutes on the treadmill was considered to have normal motor

coordination. The lower the time spent by any of the rats in walking on the rotarod treadmill, the higher the level of the motor incoordination in such test rat.

Tissue Preparation for Light Microscopy

Five rats were randomly selected from each group and were used for immunohistochemical, histochemical and histological observations. Twenty four hours after completion of treatments, the rats were anesthetized with sodium pentobarbitol and perfused through the left cardiac ventricle and ascending aorta with 4% paraformaldehyde in Tris buffer with a pH of 7.4. The brains were subsequently removed, post-fixed in the perfusate for 24 hours, and the cerebellum was excised from the cerebral hemisphere. The left half of the cerebellum of all rats were later sectioned using a cryostat, and prepared for immunohistochemical demonstration of Neuron-specific enolase (*NSE*). All stained sections were examined under Leica DM 3000 research microscope connected to a digital camera and digital photomicrographs was taken.

Statistical Analysis

The numerical data on motor coordination test were analyzed and expressed as means ± standard deviation (SD). The significant differences ($p<0.05$) in the mean values between group A and group B was checked using student's t-tests.

RESULTS

The results of the motor coordination test in the control and PbNO3 treated juvenile male rats are presented in Table 1.

Table 1: Activities of juvenile male rats treated with $PbNO_3$ for 30 days on the modified rotarod walking duration.

Group	Days					
	5	10	15	20	25	30
A (control)						
Mean (sec)	103	103	103	103	103	103
SD±	2.7	2.7	2.7	2.7	2.7	2.7
B (Lead-nitrate treated)						
Mean (sec)	100	74	60	44	40	37
SD±	7.4	9.2*	8.9*	8.5*	8.3*	12.3*

* t-test for independent samples

The motor coordination test using the modified walking rotarod indicated a significant motor incoordination in rats treated with 100 mg/kg $PbNO_3$ for 30 days ($p<0.001$). The total time spent walking on the rotarod decreased significantly in the $PbNO_3$ treated rats. After 30 days of treating the rats with 100 mg/kg body weight of $PbNO_3$, the rats could only walk on the rotarod for 47 seconds on the rotarod ($p<0.01$).

The histopathological profile of the cerebellar cortices of the rats used in this study are presented in Figures 1. The cytoarchitectural profile of the cerebellar cortex of the rats in group A (control) were with no observable neuronal disruption as seen in Fig 1A. Localized activities of *NSE* in the cerebellar cortices of the rats in the control group was observed to be low compared with the $PbNO_3$ treated rats which showed a higher neuronal immuno-positive *NSE* activities.

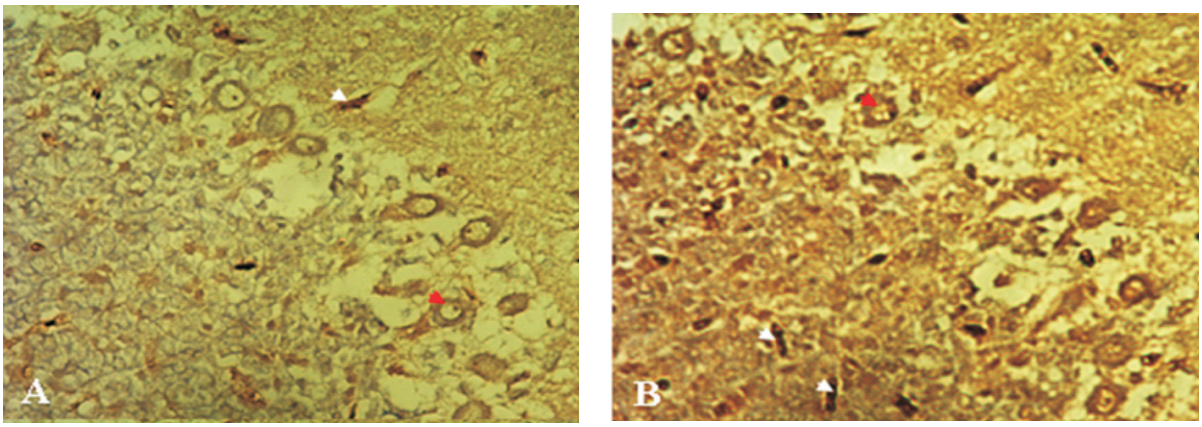

Figure 1.Effects of $PbNO_3$ treatment on the cerebellar histopathology in the rats. Rats were administered NS or $PBNO_3$ over a 30 day period and cerebellar sections were examined using immunohistochemical protocol.*A-B*) NSE at high (400x) magnification demonstrated normal pathology in NS treated rats (*A*) and higher NSE activities in the glial cells surrounding degenerated neurons in the cerebellar cortex. Photomicrographs (A and B) represent typical and representative sections from rats in each group. Note: red arrow head connotes the neurons while the white arrow head connotes the immunopositive activities of NSE signifying higher metabolic level in the glial cells surrounding the degenerating neurons which more in B

The cerebellar profile of the rats in group B treated with 100 mg/kg of $PbNO_3$ presented in Fig 1B were with degenerative characteristics such as degenerating neural connections, loss of cytoplasmic contents and nuclear materials, and increased activities of glial cells around the degenerating neurons.

DISCUSSION AND CONCLUSION

The current study was carried out to observe energy utilization in the neurons of lead-treated juvenile rats and to see if this could have any correlative effects on motor coordination in the treated animals. When we analyzed the energy utilization in the neurons of the lead-treated rats by localizing *NSE* activities in the cerebellar cortices of the rats from each groups, we noticed a significant increase in *NSE* activity in the cerebellar cortex of $PbNO_3$ treated juvenile rats.

The exposure to 100 mg/kg of $PbNO_3$ for 30 days significantly reduced the numerical population of Purkinje cells in all the lobules of the cerebellar cortices of the lead-treated rats. The brain is susceptible to the deleterious effects of free radical species as a result of its high level of oxygen metabolism and the specific composition of membranes which contain a large amount of oxidant sensitive to multiple unsaturated fatty acids. The brain has been observed to have an inadequate proportion of free radical scavenging enzymes and markers and endogenic antioxidants compared with other organs in the body [27,28].

Pb has been reported to induce oxidative damage in many organs by creating disequilibrium in the formation of reactive oxygen species. Although, the precise mechanism by which Pb bring about oxidative stress is relatively unknown.

Lead is a multipotent neurotoxin that impairs the nervous system and causes brain alterations. Although the successive use of lead has reduced, yet, lead exposure is still a health-risk because environmental lead is stable and no safe threshold for lead exposure has been established [29]. The effects of lead are particularly deleterious to the developing nervous system. According to Hsiang and Díaz [30], it is capable of causing irreversible learning and behaviour deficiencies.

In this study, it was observed that a marked statistically significant reduction in the duration the lead-treated juvenile rats spent walking on the modified rotarod after 30 days of oral exposure to $PbNO_3$. The in-ability to coordinate the anatomy of voluntary muscle movements resulting into unsteady movements and staggering gait has been reported to be one of the symptoms of lead poisoning [31]. Furthermore, ataxia resulting from lead-poisoning has been suggested to be part of the effects of Pb on glutaminegic transmission and in the impairment of executive dopaminegic functions in higher centres such motor control, attention, memory, and executive functioning which may ultimately result into numerous behavioural maladies [32].

Among the resultant neurotoxic effects of Pb is the impairment of glucose metabolism [33]. Impaired glucose metabolism is said to be the principal pathway that may be followed by alterations observed in the cerebellar neurons of the lead-treated rats as immunohistochemically localized by the activities of NSE (Fig. 1). Going by this, exposure to Pb reduced the rate of glucose metabolism, with resultant reduction of the required energy for many metabolic processes of the cerebellar neurons, and the significant decrease in the time spent in the modified rotarod by the lead-

treated juvenile rats as a result of insufficient accessible and available energy.

Lead is an environmental pollutant known to bring about a wide range of behavioural, biochemical, pathological, and physiological dysfunctions in human and animals. Results from the present study suggested that exposure to $PbNO_3$ is capable of increasing energy utilization in the cerebellar neurons in the brain of juvenile rats and also, it is capable of impairing motor coordination in the Pb exposed rats when compared with the rats in the control group treated with double-distilled water following the same protocol as in the Pb treated rats.

Neuronal apoptotic signaling pathway is often accompanied by metabolic dysregulation in the mitochondria as demonstrated in this study via immunohistochemical localization of *NSE* (Figure 1). Ogundele *et al.*[24] suggested that increased *NSE* expression is a major characteristic of neuronal cells under stress or degeneration. Increased *NSE* activities in the degenerating cerebellar neurons further supports the claim that metabolic dysregulation is involved in the neurons (Figure 1B). Several of the cells positive to the localized immunohistochemical activities of *NSE* immunostaining are cerebellar cells around the degenerating neurons.

Future studies are needed in order to observe if the alterations in the activities of *NSE* and impaired motor coordination as recorded in this study are reversible, the specific pathway between energy utilization in lead neurotoxicity and motor coordination and their associations with the subfields of the cerebellum and their relationship to cerebellar integrity in motor coordination and balancing.

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