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*Research Article*

# Behavioral and Histomorphological Changes in the Developing Brains of Vanadium-Exposed Mice Pups: Protective Role of Minocycline

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**Abstract**
Vanadium is a transition metal, abundant in nature and liberated into the environment mainly through the burning of fossils fuels, forest fires and volcanic emissions. Exposure to vanadium leads to increased reactive oxygen species (ROS) generation, lipid peroxidation and oxidative damage in the brain, with neonates and children being especially vulnerable due to an immature blood-brain barrier. Minocycline is a second-generation tetracycline antibiotic which produces neuroprotective effects in several animal models of neurological diseases, independent of its anti-microbial and anti-inflammatory properties. This study was designed to assess the behavioural and histomorphological changes in the postnatal developing mice brains after vanadium administration and ameliorating effect of minocycline. The animals were divided into 4 groups (n=12 per group): group A (Control), group B (Vanadium, 3 mg/kg body weight), group C (Vanadium 3 mg/kg body weight + Minocycline 30 mg/kg body weight), and group D (Minocycline 25 mg/kg body weight). In all groups, lactating dams were treated from postnatal day (PND) 1-14, and pups treated from PND 15-21. Open field test was conducted on PND 22 for pups before sacrifice and the brains removed for histology. Vanadium led to reduced body weight and movement, morphological changes such as depletion of pyramidal neurones in the hippocampus CA3 and cortex, central chromatolysis of Purkinje cell, gliosis, satelitosis and phagocytic nodules in the cerebral cortex. However, minocycline co-treatment exacerbated body weight loss, attenuated the movement deficits and the neuronal changes induced by vanadium. In conclusion, minocycline reduced the vanadium-induced neurotoxicity in mice pups.

**Key Words:** Vanadium, Minocycline, Central chromatolysis, Gliosis

## INTRODUCTION

Exposure to toxic metallic elements has been considered a major risk factor in the pathogenesis of chronic neurodegenerative disorders (Aschner *et al*., 2009; Kanthasamy *et al*., 2012). Early life plays an important role in health and development of an individual, therefore, exposure to toxic metals, especially in early life, is thought to mediate epigenetic changes leading to these degenerative diseases (Modgil et al., 2014). Due to immmaturity of their blood brain barrier (BBB), fetal and neonatal brains are more susuceptible to the deleterious effects of toxic metal accumulation (Bruederle and Hodler, 2019; Zheng et al, 2003). Large quantities of vanadium compounds are released into the environment mainly through the burning of fossils fuels, forest fires, volcanic emissions and formation of continental dust (Mustapha *et al*., 2014). In addition, particulate vanadium in the atmosphere as a result of increase in incidental and accidental burning of crude oil has been reported in the Nigerian Niger Delta, which is home to about 40 million Nigerians, over half of which are youths under 15 years (Azeez *et al*., 2016; Folarin *et al*., 2016).

Some studies have shown that vanadium-induced neurotoxicity led to increased oxidative stress (Bwala *et al*., 2014, and Ngwa *et al*., 2014) and neuroinflammation (Azeez *et al*., 2016) in the brain of rodents. The central nervous system (CNS) exhibits great vulnerability to oxidative stress damage, because of its continuous production of reactive oxygen species as a consequence of its high aerobic metabolism, low levels of antioxidants, and its extended surface rich in peroxidative fatty acids which has been described as an

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excellent field for neuronal injury (Wang and Michaelis, 2010).

Neuroprotection is the relative preservation of neuronal structure and/or function (Casson *et al*., 2012) and is a widely explored treatment option for many CNS disorders including neurodegenerative diseases, stroke, traumatic brain injury and spinal cord injury. Neuroprotection aims to prevent or slow down disease progression and secondary injuries, halting or at least slowing the loss of neurons (Seidi and Potashkin, 2011). Studies have shown that α-tocopherol protects the brain against vanadium-induced free radical injury (Olopade et al., 2011; Bwala *et al*., 2014). Minocycline is a second generation tetracycline antibiotic which has emerged as the most effective tetracycline derivative regarding neuroprotection (Garrido-Mesa et al., 2013). Minocycline produces neuroprotective effects in several animal models of neurological diseases like Parkinson's disease, Huntington's disease, stroke and spinal injury (Blum et al., 2004). The drug has demonstrated neuro recovery as well as neuroprotective properties (Miyoaka, 2008; Leukovitz *et al*., 2010; and Chaudhry *et al*., 2012), which are independent of its anti-inflammatory properties (Maier, 2007).

Here we determine the neurobehavioral changes and the histological changes in the brain that occur due to vanadium administration, and investigate the protective effects of minocycline on vanadium-induced neurotoxicity in mice pups.

## MATERIALS AND METHODS

**Animals:** Pregnant Swiss mice were obtained in the central animal house of the University of Ibadan and housed in the experimental animal house of the Neuroscience unit, Department of Veterinary Anatomy, University of Ibadan. The animals were fed with vital feed® and tap water ad libitum. All experiments were carried out based on the ethical guidelines of the University of Ibadan for standard care and use of animals in research.

**Experimental Design:** The pregnant mice were kept until delivery, each dam with 5-7 mice pups were housed per cage for this study. A total number of 48 pups were divided into four (4) groups (A, B, C & D), two nursing dams and their respective pups per group. Each dam had an average of six pups across all groups thus making twelve pups per group.

**Group A:** (Control group), sterile water (200 µL) was administered to the nursing dams once daily from postnatal day (PND) 1-14 intraperitoneally (IP). Sterile water (100 µL) was administered once daily to the pups from PND 15-21 IP.

**Group B:** (Vanadium group), sodium metavanadate (3 mg/kg body weight in sterile water) (Olopade et al., 2011) was administered to the nursing dams once daily from PND 1-14 IP. From PND 15-21, sodium metavanadate (3mg/kg body weight in sterile water) was administered once daily to the pups IP.

Group C; (Vanadium and Minocycline group), sodium metavanadate (3 mg/kg body weight in sterile water) was administered to the nursing dams once daily from PND 1-14 IP. From PND 15-21, sodium metavanadate (3 mg/kg and minocycline 30 mg/kg body weight) (Olopade et al., 2011 and Xue et al., 2010) were administered to the pups simultaneously once daily IP.

**Group D:** (Minocycline group), sterile water (200 µL) was administered to the nursing dams once daily from PND 1-14 IP. From PND 15-21, minocycline (25 mg/kg body weight in sterile water) (Xue et al., 2010) was administered to the pups once daily IP.

**Behavioural Tests:** The mice were weighed twice weekly using a digital weighing balance. They were tested on the open field apparatus as described by (Bwala et al., 2014) on PND 22.

The following behaviors were scored:

i. Line Crossing: Frequency with which the mouse crossed one of the grid lines with all four paws.
ii. Rearing: Number of times the mouse stood on only the two hind limbs
iii. Center Square Duration: Duration of time the mouse spends in the central square.
iv. Grooming: Sets of heterogeneous behaviours comprising face washing, body licking, paw licking, head and body shaking, scratching and genital licking

**Sample Collection and Histological Analysis:** Five mice per group were sacrificed on PND 22, after behavioral tests had been conducted. They were anaesthetized using ketamine at 100 mg/kg body weight, had transcardial perfusion with 4% phosphate buffered formalin and their brains were removed as described by Olopade et al. (2011).

The brain tissues were processed for routine paraffin embedding, and 6-7 µm thick paraffin sections were cut and stained with H & E stain for general histological examination (Folarin et al., 2017). The Nissl granules were demonstrated using Cresyl violet stain.

### Statistical Analysis

Quantitative data obtained from the behavioural test were expressed as Mean ± SEM and compared with one-way analysis of variance (ANOVA), followed by the Tukey post-hoc test, using Graphpad Prism 7.0® (2017) statistical package (San Diego, California, USA) with significance set at $p<0.05$.

## RESULTS

There was progressive body weight gain in all the mice from PND 1 to PND 21. However, there was a significant difference in the rate of weight gain across the groups (Slopes: $F(3,24)=24.3$, $p<0.0001$), with the control group having the fastest rate while the Vanadium+Minocycline group had the slowest rate of growth (Fig.1). By the end of the study, the mean body weights of the Minocycline- and Vanadium+Minocycline- treated mice was significantly less than that of the other two groups (Fig.1).

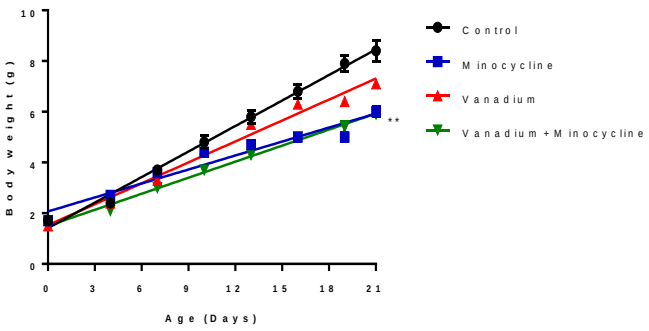


**Figure 1:**
Line graph showing the body weight measurements of the mice pups from PND 1-21 for all the groups

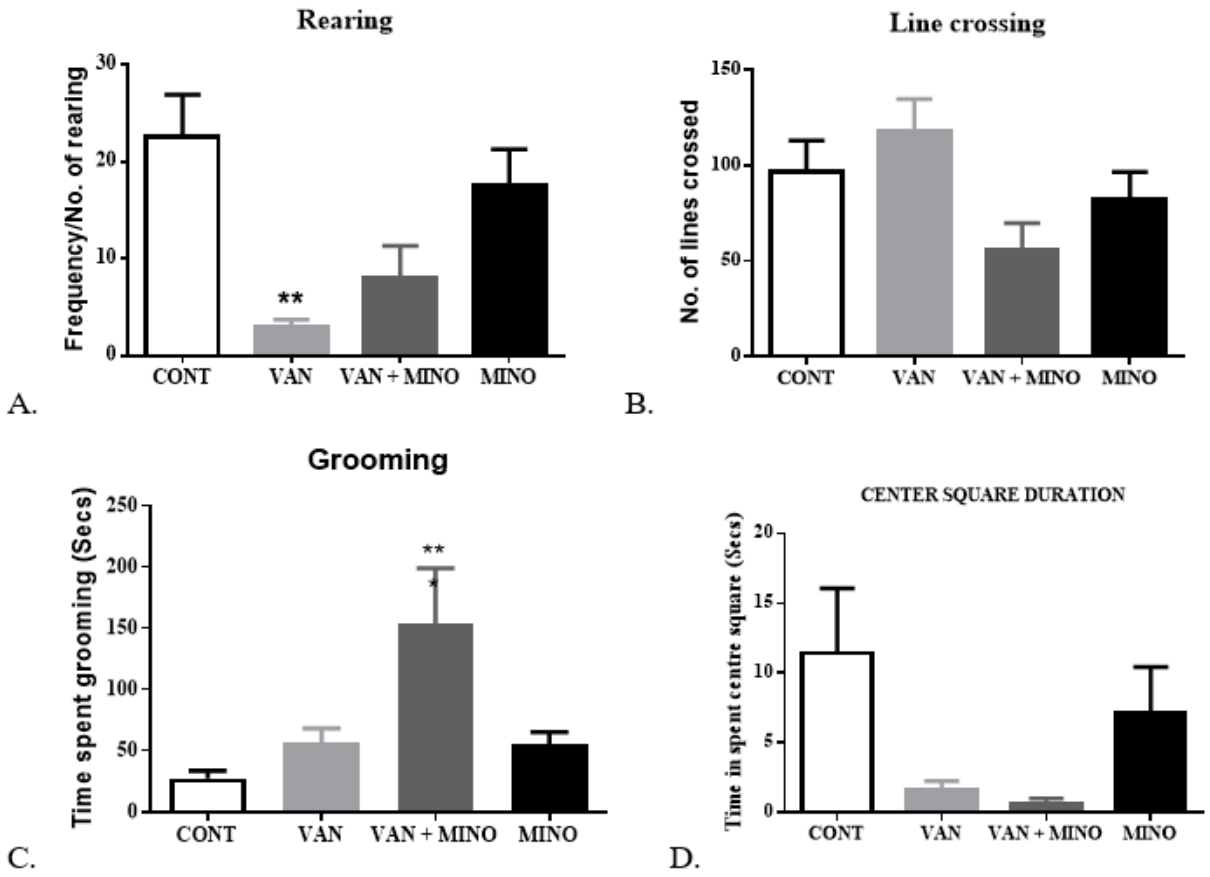


**Figure 2:**
Bar charts showing A) frequency of rearing, B) number of lines crossed, C) time spent grooming and D) time spent in the center square, across all the groups in the open field test. **p<0.01, ***p<0.001

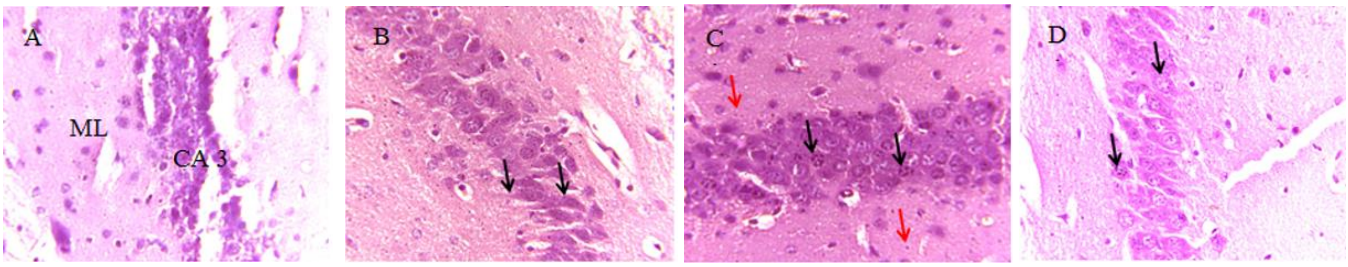


**Plate 1:**
Photomicrograph of hippocampus of control pups showing molecular layer (ML) and cornus ammonis 3 (CA3) region (A). CA3 from Vanadium group, neuronal cells are relatively few (B). CA3 from Vanadium/Minocycline group, many of the hippocampal neurons have heterochromatic nuclei (black arrows), there is mild gliosis (microglia cells) in the surrounding parenchyma (red arrows) (C). CA3 from Minocycline group, showing many hippocampal neurons with heterochromatic nuclei (black arrows) (D). A-D – X400 magnification. H&E staining.

In the open field test, the vanadium treated mice had significantly less frequency of rearing than the control and minocyline groups (p=0.0013); there was a slight increase in rearing in the Vanadium+Minocycline group, but not to significant levels. Vanadium-treated mice had increased line crossings (used as a measure of locomotion) compared to controls, while minocycline alone decreased locomotion; Vanadium+Minocycline further decreased locomotion, however, these were not to significant levels. Similarly, there was no significant difference across the groups, in the duration of time spent in the center square of the open field box, which is a measure of anxiety. However, the Vanadium+Minocycline group spent a significantly longer time grooming than all the other groups (p<0.001) (Fig. 2C).

Examination of the hippocampus in the control group revealed the molecular and pyramidal cell layers, which are deeply basophilic with heterochromatic nuclei. Following vanadium exposure, there were reduced neuronal cells in the hippocampal CA3 region. Co-treatment of minocycline/vanadium revealed mild gliosis in this study (Plate 1).

Examination of the cerebral cortex in the control group revealed the pia mater, beneath which were the six histological layers, that is; the molecular layer, the external granular layer, the external pyramidal layer, the internal granular layer, the internal pyramidal layer and the fusiform layer, the subependymal white matter and the ependymal cells lining the lateral ventricle. Vanadium administration was associated with the presence of satelitosis and phagocytic nodules in cerebral cortex. Treatment with a combination of

vanadium/minocycline resulted in milder satelitosis and fewer degenerate neurons (Plate 2 and 3A, B, C).

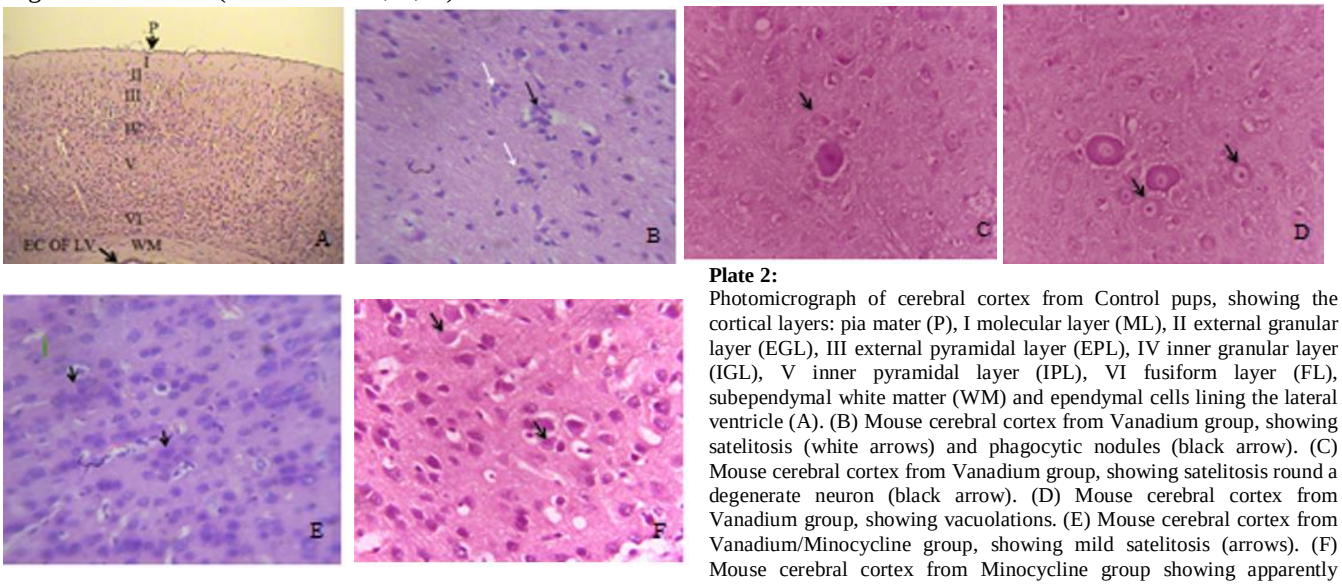


**Plate 2:**
Photomicrograph of cerebral cortex from Control pups, showing the cortical layers: pia mater (P), I molecular layer (ML), II external granular layer (EGL), III external pyramidal layer (EPL), IV inner granular layer (IGL), V inner pyramidal layer (IPL), VI fusiform layer (FL), subependymal white matter (WM) and ependymal cells lining the lateral ventricle (A). (B) Mouse cerebral cortex from Vanadium group, showing satelitosis (white arrows) and phagocytic nodules (black arrow). (C) Mouse cerebral cortex from Vanadium group, showing satelitosis round a degenerate neuron (black arrow). (D) Mouse cerebral cortex from Vanadium group, showing vacuolations. (E) Mouse cerebral cortex from Vanadium/Minocycline group, showing mild satelitosis (arrows). (F) Mouse cerebral cortex from Minocycline group showing apparently normal neuropil. A – X40 magnification, B-F – X400 magnification, H&E stain.

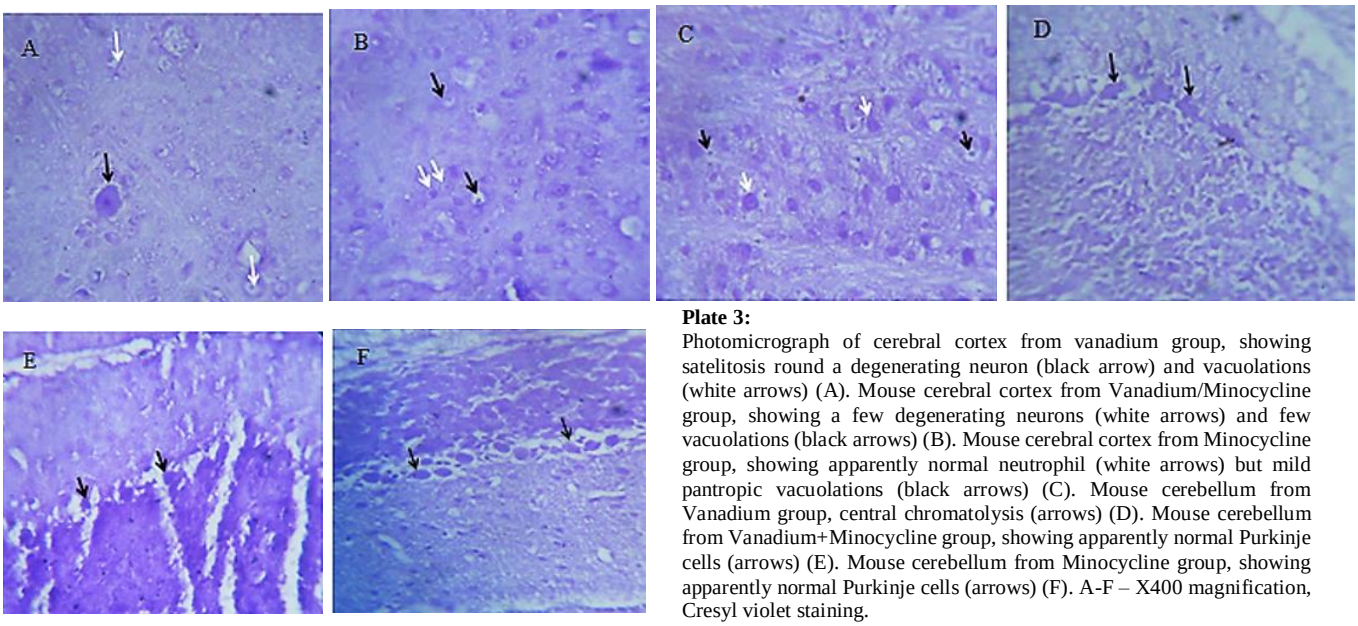


**Plate 3:**
Photomicrograph of cerebral cortex from vanadium group, showing satelitosis round a degenerating neuron (black arrow) and vacuolations (white arrows) (A). Mouse cerebral cortex from Vanadium/Minocycline group, showing a few degenerating neurons (white arrows) and few vacuolations (black arrows) (B). Mouse cerebral cortex from Minocycline group, showing apparently normal neutrophil (white arrows) but mild pantropic vacuolations (black arrows) (C). Mouse cerebellum from Vanadium group, central chromatolysis (arrows) (D). Mouse cerebellum from Vanadium+Minocycline group, showing apparently normal Purkinje cells (arrows) (E). Mouse cerebellum from Minocycline group, showing apparently normal Purkinje cells (arrows) (F). A-F – X400 magnification, Cresyl violet staining.

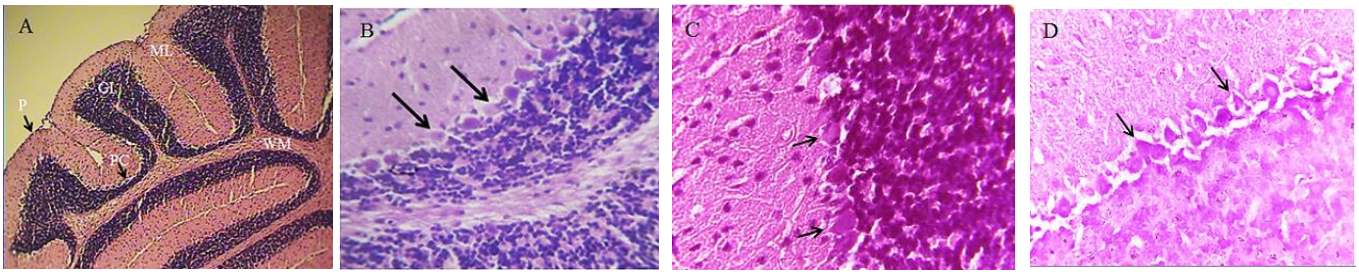


**Figure 6:**
Photomicrograph of cerebellum from Control group, Pia mater (PM), molecular layer (ML), granular layer (GL), Purkinje cells (PC) and white mater (WM) (A). Mouse cerebellum from Vanadium group, Purkinje cells with central chromatolysis (arrows) showing marginal and angulated nucleus (B). Mouse cerebellum from Vanadium/ Minocycline group, showing fewer cells with central chromatolysis (arrows) (C). Mouse cerebellum from Minocycline group, showing apparently normal Purkinje cells (D). H&E staining. A – X40 magnification, B-D – X400 magnification.

Examination of the cerebellum in the control group revealed the normal cytoarchitecture with the pia mater, outer molecular layer, Purkinje cell layer, inner granular layer, and white matter clearly seen. Vanadium administration led to development of central chromatolysis in the Purkinje cells of the cerebellum. However, in the cerebella of mice co-

administered with vanadium/minocycline there were fewer Purkinje cells with central chromatolysis (Plate 3D, E, F and Plate 4).

## DISCUSSION

This study has shown that early exposure of mice pups to vanadium led to reduced body weight, in agreement with Azeez et al. (2016) who also reported reduced body weight gain and locomotor impairment in vanadium-exposed mice. However, the combination of vanadium/minocycline further exacerbated the reduced body weight compared to the other groups of mice pups. Usende et al. (2016) reported that the combination of iron deficiency and vanadium exposure caused a reduction in body and brain weights. However, reduced body weight has not been previously reported in minocycline treatment.

The exposure of mice pups from PND 1-21 resulted in significant alterations in behavioural indices, as recorded by the open field test. Vanadium exposure led to reduced rearing, a decrease in time spent in center square but no change in grooming or frequency of line crossing in the open field box. In mice co-administrated with minocycline, rearing was partly restored but they spent less time in the center square. Manto (2012) reported that mercury, lead and other heavy metals can lead to ataxia, which is uncoordinated movement. The observed behavioural deficits are thought to be due to deficiency in motor control, which is seen in vanadium toxicity.

Following vanadium administration in the mice, we observed reduced pyramidal cells in the hippocampal CA3 region, central chromatolysis in the Purkinje cells of the cerebellum, the presence of satelitosis and phagocytic nodules in cerebral cortex. Co-treatment with minocycline however resulted in mild gliosis, fewer cells with central chromatolysis, milder satelitosis and fewer degenerated neurons. Minocycline is highly lipid soluble, easily crosses the blood brain barrier and has thus been reported to attenuate a variety of neurological disease including spinal cord injury, stroke, Parkinson's and Alzheimer's diseases (Garrido-Mesa et al., 2013). Minocycline's success in neurological diseases is proposed to be linked with its ability to inhibit microglial activation and mitochondrial release of cytochrome c into the cytosol (thus mitigating apoptosis), and suppress free radical production (Yong et al., 2004). Oxidative damage through generation of free radicals, as well as neuroinflammation are reportedly major routes of vanadium neurotoxicity (Rojas-Lemus et al., 2020; Azeez et al., 2016); it is therefore not surprising that minocycline would be of therapeutic help in this condition. In the study of Min et al. (2017), administration of minocycline to rat pups even after the onset of hypoxia (characterized by interrupted blood-brain barrier (BBB), hypomyelination and learning and memory deficits) significantly suppressed brain inflammation, demonstrating its neuroprotection in systemic hypoxia-induced brain damage.

The reduced pyramidal cells in the hippocampal CA3 region, observed in this study agrees with Folarin et al (2017), who also reported memory loss with accompanying neuronal loss in the hippocampus. Adebiyi et al (2018), similarly reported learning and memory deficits in vanadium-treated mice with associated altered myelination of hippocampal axons, which were ameliorated by administration of stigmasterol, a bioactive compound from Grewia carpinifolia. Mild gliosis observed in this study is in consonance with Simon et al (2017) who reported that treatment with minocycline reduced microglial activation in the ipsilateral cortex, hippocampus and thalamus.

Chromatolysis is a reactive change that occurs in the cell body of damaged neurons, involving the dispersal and redistribution of Nissl substance (rough endoplasmic reticulum and polyribosomes) in order to meet an increased demand for protein synthesis such as is required to regenerate axons (Moon, 2018). It is usually an early, often sublethal, change that occurs in neuronal injury, commonly the result of toxin exposure and interference with cellular metabolism. Vanadium toxicity was thus associated with this change in the Purkinje cells. Other studies have reported similar changes associated with metal toxicity. Joseph et al., (2007) observed chromatolysis in the midbrains of rabbits and lambs that have been subjected to low-dose copper toxicity. Sujatha et al. (2011), who treated rats with lead poison, observed morphological changes in the cerebellum such as spongiosis in grey matter, focal loss and shrinkage of Purkinje cells with central chromatolysis, which were ameliorated by Ocimum sanctum, a phytochemical with known anti-oxidant and anti-inflammatory properties. Minocycline, in like manner, was observed to confer neuroprotection to these Purkinje cells in this study.

Vanadium administration was associated with the presence of satelitosis and phagocytic nodules in cerebral cortex, similar to Ghazaryan et al. (2017) who reported moderate hypertrophy of the surviving pyramidal cells, satelitosis with lysis in rats that had neuronal damage induced with madopar (L-dopa). Treatment of vanadium-exposed mice with minocycline resulted in mild satelitosis and fewer degenerating neurons. Minocycline's neuroprotective ability has also been demonstrated by restoration of neuronal metabolism, decrease in astrogliosis, microglial activation, and neuronal loss in macaques infected with simian immunodeficiency virus (Ratai et al., 2010).

A number of compounds have been proposed and reported to provide a level of protection against vanadium neurotoxicity, these include Vitamin E (Olopade et al., 2011), kolaviron (Igado et al. 2017), Moringa oleifera leaves (Igado et al., 2017) and Erythropoietin (Mustapha et al., 2014), but each with its limitations. To the best of our knowledge, this is the first report of minocycline being evaluated used in this condition, and it has shown some protective potentials.

In conclusion, this study showed that vanadium toxicity led to locomotor impairment, death of pyramidal neurons in hippocampus CA3 region, central chromatolysis of Purkinje cells in cerebellum, satelitosis and phagocytic nodules of cerebral cortex. Minocycline treatment exerts some neuroprotective effect on vanadium-induced neurotoxicity, being effective in some sections of the brain but mild in others. It however resulted in body weight reduction, especially when given in combination with vanadium

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