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Original Article

Thalamic Immunohistochemical Assessment in Wistar Rats Following Combined Exposure to Nickel and Vanadium

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Abstract

BACKGROUND AND AIM: Nickel (Ni) and vanadium (V), major constituents of crude oil, have been shown to induce neurotoxic responses. However, there is paucity of reports on the impact of their combined exposure and likely potentiating consequences. This study aimed to assess the effect of Ni and V co-exposure on the thalamus of rats, by evaluating immunohistochemical markers of brain integrity including glial fibrillary acidic protein (GFAP), ionized calcium-binding adapter molecule 1 (Iba-1), neuronal nuclei antigen (NeuN), nuclear factor erythroid 2-related factor 2 (Nrf2) and parvalbumin protein.

METHODOLOGY: Twenty-four adult Wistar rats were randomly divided into four groups: saline only (Control), 20 mg/kg Ni orally for 21 days, 3 mg/kg V intraperitoneally for 7 days and combined Ni and V treatments.

RESULTS: Results showed significantly increased expression of GFAP, Iba1 and NeuN in all treatment groups. However, there was consistently marked alterations with Ni treatment compared to control with V exposures appearing to attenuate Ni impact for combined exposures. Additionally, increased Nrf2 immunoreactivity and decreased parvalbumin immunoreactivity were observed in all treatment groups compared to control.

CONCLUSION: Overall, the study demonstrates that while both Ni and V can cause toxicity in the thalamus, combined exposure showed opposing effects of their co-accumulation in the thalamus which suggests that V treatment could mitigate the Ni-induced thalamic neurotoxicity.

Keywords:
neurotoxicity, metals, thalamus, neuroinflammation, oxidative stress.

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INTRODUCTION

Over the last decade, heavy metals have been implicated in the onset of neurodevelopmental and neurodegenerative diseases by various studies on metal neurotoxicity, and with the spread of industrialization towards residency, the risk of metal intoxication increases (Adebiyi *et al.,* 2018; Ijomone *et al.,* 2018). Exposure to these metals is inevitable as they are resourceful materials for construction, producing kitchenware, prosthetics, automobile engineering and just about every machinery in our world today, and as such, we encounter them on a daily basis (Mahmoud *et al.,* 2011). Compounds containing heavy metals have been shown to cause noxious conditions (Aljelehawy 2022). In the nervous system, some divalent heavy metals compete with functional divalent ions, thereby disrupting cell metabolism and homeostatic mechanisms ultimately (He *et al.,* 2020).

Nickel (Ni) is a divalent metal which is transported by the divalent metal transporter (DMT-1) across cells and promote the formation of free radicals within the brain (Son 2020). Accumulation of Ni within the nervous system has been associated with the onset of neurodegenerative diseases following the formation of α–synuclein amyloid (Li *et al.,* 2021). Vanadium (V), on the other hand, is a transition element which is transported by albumin and transferrin proteins.

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The latest use of vanadium involves green technologies and the production of vanadium-based redox flow batteries, these very efficient and increasingly popular energy storage systems have already been installed, in the following countries: China, the USA, Germany, and Japan. The industrial use of vanadium is on the increase and so is the release of vanadium to the environment (Zwolak 2020). While Ni is mainly absorbed by the body via food and water intake, inhalation is the most common route of exposure for V compounds (Genchi *et al.,* 2020; Rojas-Lemus *et al.,* 2021). In a recent study, it was noted that vanadium entered the body by binding to particulate matter, an atmospheric mixture of organic and inorganic masses, which readily translocate through the alveoli of the lungs due to their micro-diameter (<2.5µm) (Rojas-Lemus *et al.,* 2021).

Typically, metal neurotoxicity is characterised by abnormal levels of reactive oxygen species (ROS), inflammatory cell activity, damage to cell survival mechanisms and a few injurious evidence within the affected regions. Inflammatory markers like glial fibrillary acidic protein (GFAP) and ionized calcium-binding adapter molecule 1 (Iba-1) are often targeted using corresponding antibodies to monitor the activities of inflammation-responsive glial cells (astrocytes and microglia) post-trauma. In addition, neuronal nuclear protein (NeuN), nuclear factor erythroid 2-related factor 2 (Nrf2), and parvalbumin (PV) antibodies are employed for examining alterations in the neuronal populations, oxidative stress regulation and GABA inhibitory activity, respectively (Neri *et al.,* 2018; Lin *et al.,* 2021; Zhang *et al.,* 2021).

While some studies have been undertaken to understand the impact of these metals on several regions of the brain, there is limited account on the outcome of concurrent exposure of Ni and V to the nervous system. In this study, the thalamus was the focal region of study, as it plays a key role in information processing within the central nervous system. It primarily serves as a relay centre for information transfer between the higher centres and the brainstem with exception to olfaction. Functionally, the thalamus is subdivided into various nuclei, consisting of packed excitatory and inhibitory neurons, responsible for transmitting specific sensory signals to corresponding cortical areas (Sherman 2016; Habas *et al.,* 2019).

This study was undertaken to discover the effects of the combined exposure to nickel and vanadium on the thalamus because of their presence in crude oil, a valuable and highly exploited energy source that can be found in the Niger Delta region of Nigeria, where the ceaseless occurrence of crude oil pollution, has led to the deterioration of the ecosystem and rendered the area barely habitable (Akinwumiju *et al.,* 2020). Here, we evaluated the impact of Ni and V on the thalamus using immunohistochemical staining techniques for GFAP, Iba1, NeuN, Nrf2 and PV.

MATERIALS AND METHOD

Animal care and treatment

Twenty-four adult male Wistar rats, with weights ranging between 150g and 200g, were used for this study. The rats were housed in clean plastic cages and allowed access to food and water *ad libitum*. All experimental protocols were in strict accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011), and approved by the Institutional Research Ethics Committee (FUTA/ETH/23/97).

Rats were randomly assigned into four groups of six animals each and treated as follows:

- Control: Received normal saline (vehicle for Ni and V) orally for 21 days.
- Ni only: received Ni at 20 mg/kg per day orally for 21 days.
- V only: received 3 mg/kg of V per day via intraperitoneal injections for 7 days.
- Ni + V: received Ni as above for 21 days in combination with V as described above for the last 7 days.

Ni was administered as nickel chloride ($NiCl_2$ – Sigma-Aldrich, USA) and V was administered as sodium metavanadate ($NaVO_3$ – Sigma-Aldrich, USA). Doses of Ni and V were based on previous studies (Olopade *et al.,* 2011; Ijomone *et al.,* 2018). Once the administration period elapsed, rats were euthanized with isoflurane inhalation, brains were promptly removed and fixed in 10% neutral buffered formalin for immunohistochemical evaluation.

Immunohistochemistry

Fixed brains were prepared for standard paraffin embedding and 5µm thin sections were cut on a rotary microtome to reveal the thalamic regions. This was achieved via mid-sagittal sections at ~ lateral -2.00 to 2.40 mm to midline, using the rat brain atlas a guide (Paxinos and Watson 2007) (see Figure 1). A heat-induced antigen retrieval technique, using a citrate-based unmasking solution, pH 6.0 (Vector®, Burlingame, CA, USA; #H3300) was performed on deparaffinised slides, as slides were heated for about 30 minutes and subsequently cooled at room temperature for 30 minutes. For the next 10 minutes, the slides were submerged in an endogenous peroxidase blocking solution of 0.3 % hydrogen peroxide in Phosphate Buffered Saline. Sections were then incubated for 2 1/2 h at room temperature in primary antibodies diluted in a universal antibody diluent and blocking reagent, UltraCruz® Blocking Reagent (Santa Cruz, USA). Antibody incubation protocols utilised: GFAP (ThermoFisher, USA; #16825-1-AP) at 1:7500, IBA1 (Cell Signaling, USA; #17198) at 1:1250, NeuN (ThermoFisher, USA; #26975-1-AP) at 1:1500, Nrf2 (ThermoFisher, USA; #PA1-38312) at 1:100, Parvalbumin (Novus Biologicals, USA; NB120-11427) at 1:1000. After washing, sections were incubated in ImmPRESS™ (Peroxidase) Polymer Anti-Rabbit IgG Reagent, made in horse

(Vector® #MP-7401). Brown immunoreactive colour was developed with DAB Peroxidase (HRP) Substrate Kit (Vector® #SK-4100), and sections were counter-stained in haematoxylin.

Photomicrography and quantification

Immunostained slides were digitized with the Panoramic 250 Flash II slide scanner (3D Histech, Budapest, Hungary) as shown in figure 1. A complementary digital microscopy software, Caseviewer, was employed in the random capturing of Eight to ten parallel photomicrographic fields of the thalamic regions. Thereafter, analysis of photomicrographs was done using Immunoratio and cell count plugins of the NIH-sponsored ImageJ software. The ImmunoRatio plugin estimates the ratio of brown DAB (positive immunoreactivity) to the haematoxylin counterstain by digital colour deconvolution. The cell counter plugin records the number of manually selected cell types (Erukainure *et al.,* 2019; Akingbade *et al.,* 2021). The average scores of analysed photomicrographs were used for data analysis.

Statistical analysis

Data analysis was carried out with GraphPad Prism Version 8 (GraphPad Inc, San Diego, US) statistical software to run a One-way ANOVA followed by Tukey's multiple comparison test. Statistical significance was set to $P < 0.05$.

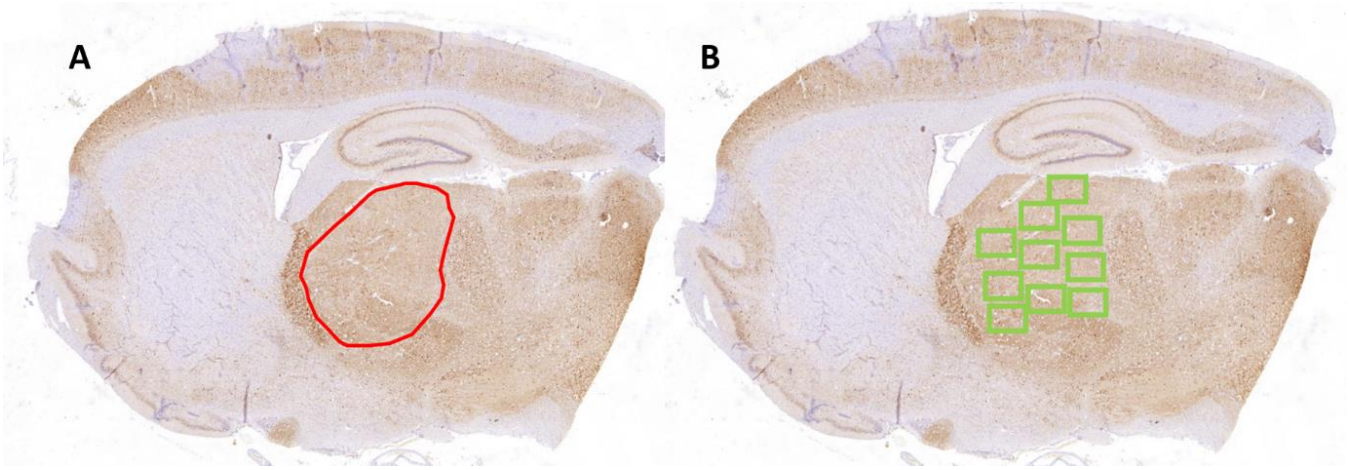

Figure 1: (A) Mid-sagittal section (~ lateral -2.00 to 2.40 mm to midline) of rat brain showing the thalamus in red annotation. (B) non-overlapping annotations of the thalamus (green), randomly selected for image analysis and quantification of immunoreactivity

RESULTS

Elevated GFAP expression in the thalamus following nickel and vanadium exposure.

Cell count analysis showed significant increase ($P<0.05$) in the GFAP immunopositive cells within the thalamus of rats treated with nickel [17.91±0.59], vanadium [12.64±0.25] and nickel + vanadium [15.20±1.02] compared to control [9.36±0.37]. The nickel group showed a significant increase ($P<0.05$) in GFAP immunopositive cells compared to the nickel + vanadium group. Cell count analysis within the thalamus of rats treated with vanadium was significantly decreased ($P<0.001$) compared to the nickel-treated group.

In addition, a significant increase ($P<0.01$) in GFAP expression was noted in the metal-treated groups compared to the control group. Nickel [25.99±0.41], vanadium [13.33±0.77] as well as in the nickel + vanadium [22.65±0.48], increased GFAP expression levels when compared to control [10.18±0.34]. In addition, GFAP expression was significantly increased ($P<0.01$) in the nickel group and significantly reduced ($P<0.01$) in the vanadium group compared to the nickel + vanadium co-exposure group. Furthermore, GFAP expression in the group treated only with vanadium was significantly reduced ($P<0.001$) when compared to the nickel group or the co-treated group.

Increased Iba1 expression in the thalamus following nickel and vanadium exposure.

Cell count analysis showed significant increase ($P<0.001$) in Iba1 immunopositive cells across all treated groups: nickel [14.72±0.21], vanadium [10.86±0.36] and nickel + vanadium [9.63±0.31] when compared to the control [6.50±0.34]. More so, there was a significant increase ($P<0.001$) in the cell count of the nickel group when compared to both the nickel + vanadium group and the group treated with only vanadium. Iba1 immunoexpression analysis showed a significant increase ($P<0.001$) in all groups treated with metals. Nickel [20.85±0.43], vanadium [18.53±0.02], nickel + vanadium [19.37±0.33], significantly increased Iba1 immunoexpression compared to the control [10.73±0.3846].

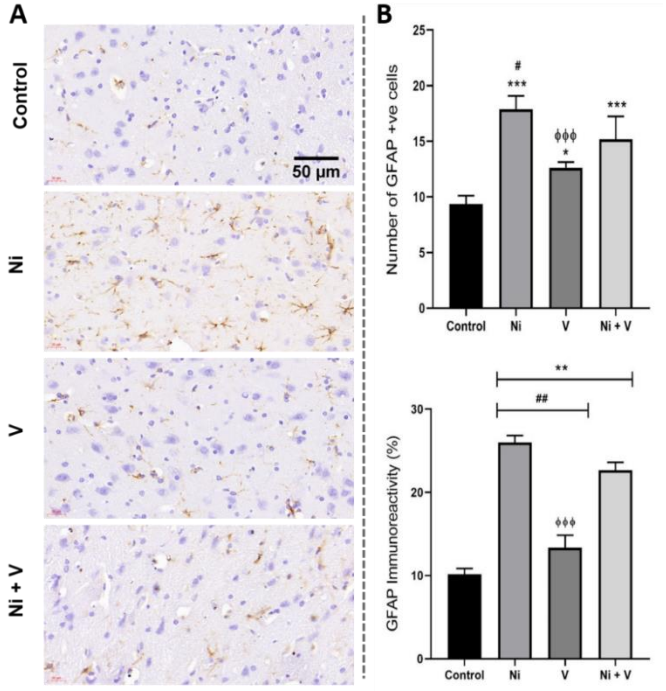

Figure 2: (A) Photomicrographs (Mag = 400X) showing GFAP immunohistochemistry within the thalamus of rats treated with Ni and V. (B) Bar chart of the mean with SEM demonstrating the quantitative analysis of GFAP immunoreactivity and cell count. Comparisons to the control group (*P<0.05), Ni + V group ($^{\#}$P<0.05) and Ni group ($^{\Phi}$P<0.001).

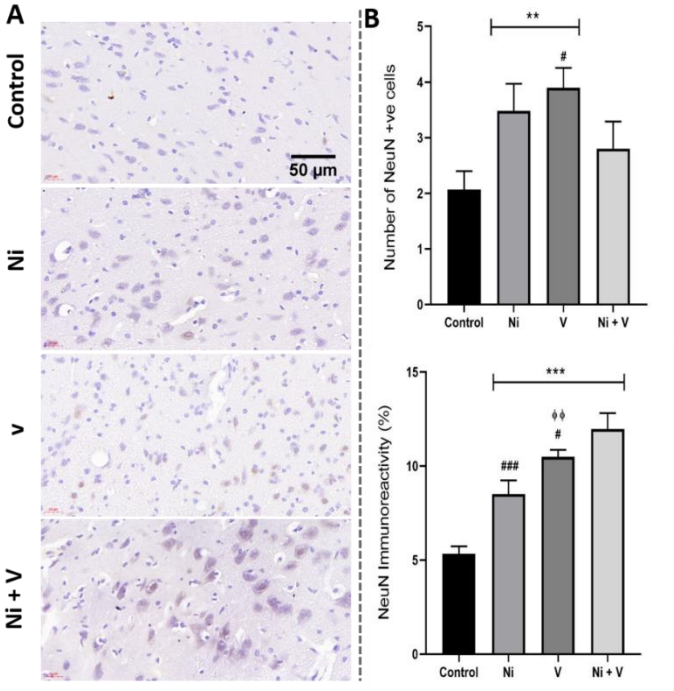

Figure 4: (A) Photomicrographs (Mag = 400X) showing NeuN immunohistochemistry within the thalamus of rats treated with Ni and V. (B) Bar chart of the mean with SEM illustrating the quantitative analysis of NeuN immunoreactivity and cell count. Comparisons to the control group (*P<0.01), Ni + V group ($^{\#}$P<0.05) and Ni group ($^{\Phi}$P<0.01).

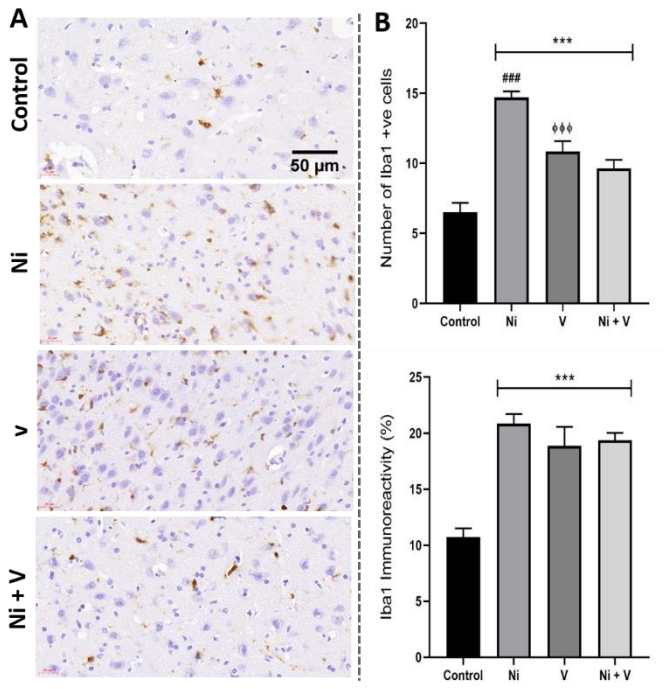

Figure 3: (A) Photomicrographs (Mag = 400X) showing Iba1 immunohistochemistry within the thalamus of rats treated with Ni and V. (B) Bar chart of the mean with SEM demonstrating the quantitative analysis of Iba1 immunoreactivity and cell count. Comparisons to the control group (*P<0.001), Ni + V group ($^{\#}$P<0.001) and Ni group ($^{\Phi}$P<0.001).

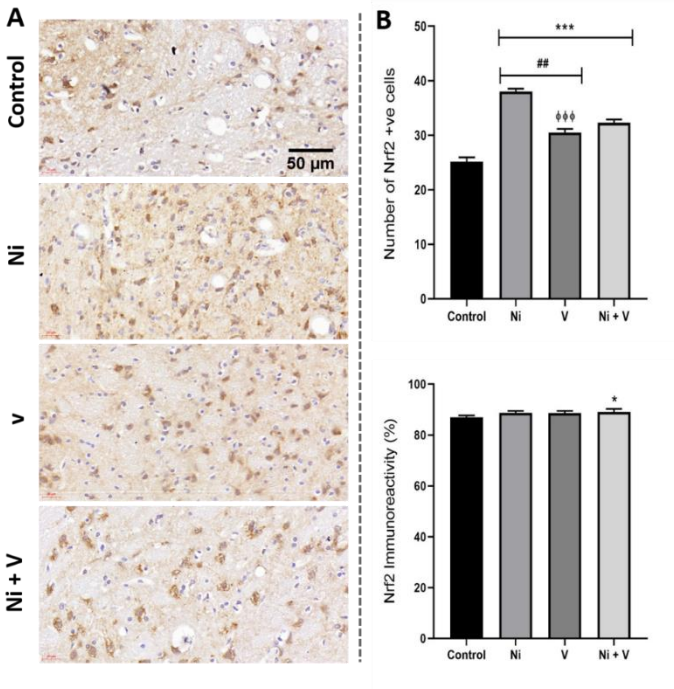

Figure 5: (A) Photomicrographs (Mag = 400X) showing Nrf2 immunohistochemistry within the thalamus of rats treated with Ni and V. (B) Bar chart of the mean with SEM illustrating the quantitative analysis of Nrf2 immunoreactivity and cell count. Comparisons to the control group (*P<0.05), Ni + V group ($^{\#}$P<0.01) and Ni group ($^{\Phi}$P<0.001).

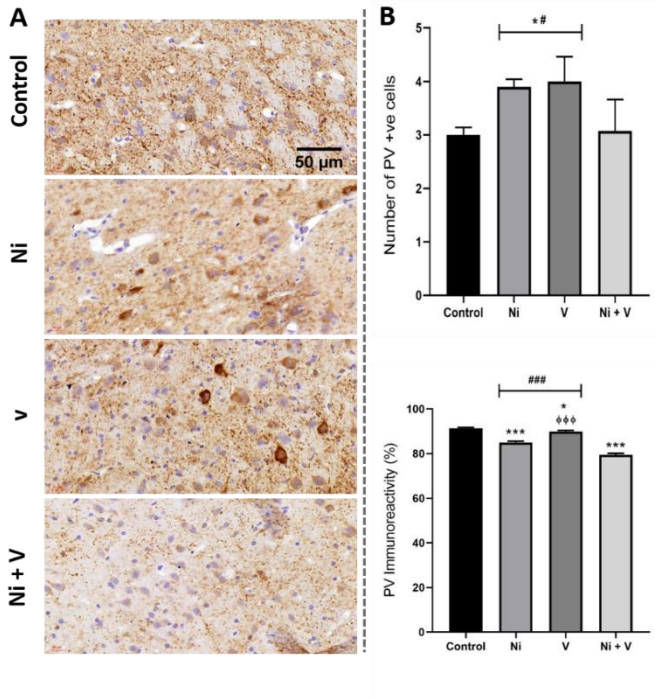

Figure 6: (A) Photomicrographs (Mag = 400X) showing PV immunohistochemistry within the thalamus of rats treated with Ni and V. (B) Bar charts of the mean with SEM illustrating the quantitative analysis of PV immunoreactivity and cell count. Comparisons to the control group (*P<0.05), Ni + V group (#P<0.01) and Ni group (ΦP<0.001).

Increased NeuN expression in the thalamus following nickel and vanadium exposure.

Cell count analysis revealed significant increase (P<0.01) in the population of NeuN immunopositive cells in groups treated with nickel [14.72±0.2095] and vanadium [10.86±0.3607] compared to the control [6.500±0.3367]. In addition, there was a significant increase (P<0.05) in the cell count of the vanadium group when compared to the group treated with nickel. Results from the NeuN immunoexpression analysis showed significant increase (P<0.001) in all groups treated with metal: nickel [8.507±0.3650], vanadium [10.50±0.1780], nickel + vanadium [11.97±0.4226], compared to the control [5.331±0.2005]. NeuN immunoexpression show significant increase (P<0.05) in the nickel group and the vanadium group compared to the nickel + vanadium group. A significant increase (P<0.001) in NeuN expression was noted in in vanadium-treated rats compared to the nickel group.

Elevated Nrf2 expression in the thalamus following nickel and vanadium exposure.

Cell count analysis revealed significant increase (P<0.01) in the number of Nrf2 immunopositive cells in groups treated with nickel [38.00±0.27], vanadium [30.53±0.31] and nickel + vanadium [32.29±0.30] compared to the control [25.16±0.39]. In addition, a significant difference (P<0.01) was noted in cell count in the nickel and vanadium groups when compared to the nickel + vanadium group. Cell counts in the vanadium group was significantly decreased (P<0.001) compared to the nickel-treated group. Results from the Nrf2 immunoexpression study showed significant increase (P<0.05) in the nickel + vanadium [89.05±0.60] compared to the control group [86.91±0.40]. However, there are no significant changes between the nickel-treated group [88.72±0.37] and the vanadium group [87.34±0.41] when compared with the control group, nickel + vanadium group or against each other.

PV expression in the thalamus following nickel and vanadium exposure.

Results from the parvalbumin immunoexpression analysis showed a significant decrease (P<0.05) in all metal-treated groups: nickel [85.00±0.30], vanadium [89.83±0.30] and nickel + vanadium [79.45±0.32] compared to the control group [91.32±0.29]. Also, there are significant increments (P<0.001) in thalamic PV expression of rats treated solely with either nickel or vanadium when compared with the group co-treated with both metal compounds. Furthermore, there is a significant increase (P<0.001) in PV immunoexpression in the group treated with vanadium when compared to the nickel-treated group. Cell count analysis revealed significant increase (P<0.05) in the cell population in groups treated with nickel [3.90±0.07] and vanadium [4.00±0.23] compared to the co-treated group [3.08±0.29] and the control group [3.00±0.07]. There was no significant difference (P<0.05) in the cell population of the co-treated group when compared to the control.

DISCUSSION

Physiologically, essential trace metals in minute quantities function to promote enzymatic activities. However, the buildup of these metals may alter homeostatic conditions. Insults from heavy metal accumulation within the nervous system have been shown to disrupt neurotransmission, evoke neuroinflammatory responses, increase oxidative stress, promote DNA damage, alter nervous tissue morphology and even lead to cell death (Adebiyi *et al.,* 2018; Ijomone *et al.,* 2020a; Ijomone *et al.,* 2020b). While brain regions like the cerebral cortex, the substantia nigra and the hippocampus are common foci for metal neurotoxicology, the thalamus should also be considered as a critical region for evaluation as it is heavily involved in neurotransmission within the nervous system (Afifi and Embaby 2016; Bouabid *et al.,* 2016; Sherman 2016; Karri *et al.,* 2018). In view of this, the present study assessed neuroinflammatory responses by evaluating astrocytic and microglia responses, antioxidant regulation and neuronal excitability modulation following Ni and V exposure in rats' thalamus.

Here, we show increased GFAP immunopositive cells as well as increased immunoexpression in the thalamus of rats treated with these metals. This is consistent with previous studies in which astroglia upsurge was observed upon

exposure to heavy metals (Erazi *et al.,* 2010; Rai *et al.,* 2013; Varmazyari *et al.,* 2020). The former study included the administration of aluminum chloride to rats *in utero* and 4 months postpartum which increased GFAP expression in the cerebral cortex. While generally affording neuroprotection, astrocytes are also known to mediate repair post insult, which may very well be the case here. Similarly, increased thalamic Iba1 expression was recorded herein. Primarily, Iba1 serves as a regulatory protein for the immune responsive function of active microglial cells under neuroinflammatory conditions. A recent study showed that lead and cadmium exposure elevated Iba1 expressions in the dentate gyrus (DG) of the hippocampus (Gök and Deveci 2022). As such, increased Iba1 expression within the thalamus of rats treated with Ni and V is consistent with neuroinflammatory responses (Norden *et al.,* 2016; Tsai *et al.,* 2021). Furthermore, it was observed that combined treatment with Ni and V yielded relatively lower astrocyte and microglial activation compared to individual exposures of both metals. This could be attributed to the antagonistic relationship between Ni and V as described by (Dreher *et al.,* 1997; Kodavanti *et al.,* 1997; Mahmoud *et al.,* 2011). It has been suggested by earlier studies that vanadium, in low concentration, plays a neuroprotective role against oxidative stress and inflammation , which correlates with the nickel-vanadium antagonistic relationship earlier inferred as nickel is a recognized promoter of the aforementioned forms of toxicity (Liu *et al.,* 2017; Ijomone 2021; Semiz 2022; Femi-Akinlosotu *et al.,* 2023). Additionally, Ni administration induces relatively higher immunopositivity, which is consistent with a previous study where lactose dehydrogenase (LDH), an inflammatory marker, was substantially elevated in Ni and copper (Cu) compared to other heavy metals (Rice *et al.,* 2001).

Immunohistochemistry revealed an increased number of NeuN-positive cells in the thalamus of rats treated with Ni and V. The NeuN marker localizes mature neurons containing the neuronal nuclear antigen. This localization has been previously shown to be specific to neurons, however, a recent cell culture study posited that astrocytes may also possess this neuronal antigen (Darlington *et al.,* 2008; Gusel'nikova and Korzhevskiy 2015). Thus, the observed increase in NeuN positivity within the thalamus of treated rats may represent astrocytic recruitment, corresponding to the rise in GFAP expression.

Nrf2 protein has been implicated as a promoter of the anti-oxidative defense system in a previous study (Ma 2013). Here we noted slight disparities in Nrf2 expression within the thalamus across all metal-treated groups, which may reflect low concentration or exposure time necessary for the detachment of Nrf2 from Keap1 (kelch-like ECH-associated protein 1) (Taguchi *et al.,* 2011).In addition, while there is an increase in the number of Nrf2 positive cells within the thalamus of metal-treated groups, indicative of alterations in the oxidative balance, there is a relatively lower Nrf2 expression in the co-treated groups. This may be due to the antioxidative effect of vanadium administered in trace quantities as shown in previous studies where it restored hippocampal neuroarchitecture (Sury *et al.,* 2011), possibly by upregulating pro-survival pathways and down-regulating phosphatase and tensin homolog (PTEN) (Tsave *et al.,* 2016; Femi-Akinlosotu *et al.,* 2023).

PV interneurons which are majorly GABAergic, play a vital role in excitatory-inhibitory balance, as such, a disruption in this functionality would precede the onset of neurological disorder (Gao and Penzes 2015; Berkowicz *et al.,* 2016). Although there was modest increase in the PV positive cells following metal treatments, our findings revealed marked decline in parvalbumin expression following Ni and V administration. The observed decrease in PV concentration is expected as similar occurrences have been implicated in a study that noted the appearance of schizophrenic-like behavioral changes, including elevated locomotor and anxiety-related activities (Abazyan *et al.,* 2014), possibly influenced by alterations in the function and execution of N-methyl D-aspartate receptors (NMDAR) after lead treatments (Opler *et al.,* 2008; Abazyan *et al.,* 2014).

In conclusion, nickel and vanadium administration evoked microglial and astrocyte activation, neuronal perturbations, Nrf2 activation, and disruption of parvalbumin excitatory-inhibitory balance. Furthermore, the co-administration of both metal compounds showed opposing effects of their co-accumulation within the thalamus across this study, hence, relatively lower indices compared to administration of individual metal compounds. Nonetheless, further investigations into the relative thalamic affinity for both metals and their contribution to neurotoxicity should be carried out, to better characterize their mechanisms of action.

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Conflict of interest

The authors declare no conflict of interests.

Author contribution

Conceptualization – OMI; Study design – OKI and OMI; Animal experiments – GEE, OIO, HOE; Immunohistochemistry, image acquisition and data analysis – GEE, OIO, OKI, OMI; Manuscript draft – GEE, OIO; Critical revisions and editing – OMI. All authors approved the final draft.

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