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Original Article
Immunohistochemical Investigations of the Thalamic Region in Rats Following Combined Exposure to Metals and Restraint Stress

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ABSTRACT

Chronic exposure to stress has been linked to the perturbation of normal brain functioning. Further, metal overexposure due to their increasing applications can negatively impact brain health. Combined exposure to stressful events and metals is common but has not yet been studied. Thus, this study focused on the thalamic region for its role as a vital relay station in the brain to investigate apoptotic and microglia activation, oxidative stress regulation, and myelin damage following co-exposures to restraint stress and metals, manganese (Mn) and nickel (Ni). Thirty-six adult male rats were divided into six groups and, respectively, exposed to the following for 15 days: control group (normal saline), stress group (3 hours of restraint stress daily), Mn and Ni only groups (intraperitoneal injection of 25 mg/kg of metals), and stress + metal groups received Mn or Ni prior to being subjected to restraint stress. Following treatments, immunohistochemical procedures were used to evaluate relevant neurochemical markers. Results show significantly increased activation of caspase-3 in stressed, metal-only, and combined stress-plus-metal treatments, particularly with Mn treatments. Also, the results show a varying response to Iba1, the microglia activation marker. Furthermore, the study reports significant decreases in Nrf2 (nuclear factor erythroid 2-related factor 2) expression that is potentiated by combined stress and metal treatments as well as altered myelination-linked proteins, Olig2 (oligodendrocyte lineage transcription factor 2), and MBP (myelin basic protein). Overall, the result from this study indicates that the combination of stress and metal exposure could exacerbate the neurotoxic impact of metal toxicity or stressful events.

Keywords
Chronic stress, Neurotoxicity, Manganese, Nickel

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INTRODUCTION

Urbanisation and industrialization have both contributed to an overall increased prevalence of heavy metals in the environment, threatening the ecosystem's natural balance (Aliyev et al. 2018). Among many metals present in the environment, Mn and Ni are important metals with various industrial applications; both metals are used in the production of steel, dry batteries, and household and cooking utensils (Gong et al. 2017; Khan et al. 2022). Research carried out on the brains of industrial workers that utilised Mn demonstrated high Mn accumulation in several brain regions (Finkelstein et al. 2008; Long et al. 2014), thus altering neurophysiology and consequently neurodegeneration (Caito and Aschner 2015). Mn toxicity is facilitated by the generation of reactive oxygen species and the depletion of the cellular antioxidant defence mechanism (Martinez-Finley et al. 2013). It is established that Mn and Ni are deleterious to brain health and employ neurotoxic mechanisms such as the generation of free radicals leading to oxidative stress (Ijomone et al. 2020a; Ijomone et al. 2020b) and apoptosis (Tinkov et al. 2021). Human exposure to Ni is mainly via oral, dermal, or nasal routes (Ijomone et al. 2018a). There has been no evidence

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to denote the nutritional benefit of Ni in humans (Song et al. 2017). Overexposure to Ni has been shown to result in toxicities of the different organs, including the brain; it affects cognitive functions, motor, and mood behaviours (Ijomone 2021).

Stress, which has been described as any state that results in disconcertment of the homeostatic or normal physiological condition of the body (McEwen et al. 2016) is part of our daily lives. Acute stress, termed "eustress," can be beneficial as it plays a vital role in improving learning and memory abilities (Lindau et al. 2016; Cacha et al. 2019); whereas, persistent stressful events produce a state of chronic stress that is a risk factor for the onset and/or progression of mental health issues due to the ability of stress hormones (corticosteroids in rodents and cortisol in humans) to accumulate in the brain and influence cognition as well as affective processing (Lupien et al. 2018). Chronic stress induces elevated levels of glucocorticoids in the body, resulting in structural alterations in the limbic regions, including the hippocampus, prefrontal cortex, and amygdala, and consequent behavioural anomalies and psychopathologies (Dagnino-Subiabre et al. 2006; Lupien et al. 2018). Stress also results in a series of physical manifestations, including hypertension, tachycardia, and slower digestion (Cacha et al. 2019). Early life exposure to stress may affect brain development and maturity, eliciting inflammatory responses from exposure to certain environmental pollutants later in life. Recent research has linked this inflammation to the aetiology and progression of mental and neurodegenerative diseases (Solarz et al. 2023). The thalamus has been shown to coordinate stress responses from the external environment (Rowson and Pleil 2021). The thalamus has a functional connection to the hippocampus, basal ganglia, and cerebellum and is believed to process sensory information as well as relay it to the brain's cortical motor areas (Georgescu et al. 2020).

Over 70% of chronic disease risks are probably due to environmental factors (Rappaport and Smith 2010; Weaver et al. 2022). A combination of several environmental factors may pose even greater consequences, particularly for neurological deficits. The combined impact of stress and metal exposures is yet to be elucidated; hence, we investigated the effect of such a combination, focusing on restraint stress in combination with Mn or Ni exposures, on thalamic apoptotic activities, microglial activation, and myelin integrity

MATERIALS AND METHODS

Animal models and treatments

Thirty-six male Wistar rats (150–200 g) were used for this study. Rats were housed in clean, square-shaped, transparent cages with a wire mesh top and floored by sawdust with a natural daylight cycle. All rats had free access to standard laboratory rat chow and water *ad libitum.* All experimental protocols were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals (National Research Council 2011) and approved by the Institutional Research Ethics Committee (FUTA/ETH/23/99).

The animals were randomly divided into six groups of six rats, as follows: The control group received normal saline, a vehicle for metals; the stress group was subjected to 3 h of restraint stress daily; Mn and Ni only groups received 25 mg/kg metals; stress in combination with metals groups received Mn or Ni before being subjected to restraint stress 30 min after administration. Restraint stress was performed daily for 15 days by immobilising animals in a narrow transparent glass chamber measuring 24 × 6 cm, as previously described (Lapmanee et al. 2017). Mn and Ni administrations were via intraperitoneal injections once every two days for 15 days, for a total of eight injections. Mn was administered as manganese chloride ($MnCl_2$, Sigma-Aldrich, USA) and Ni as nickel chloride ($NiCl_2$, Sigma-Aldrich, USA). The dose and mode of metal administration are based on our previous studies (Akingbade et al. 2022; Ijomone et al. 2022). At the completion of the treatment period, rats were euthanized via isoflurane inhalation, and the brains were rapidly excised and fixed in 10% neutral buffered formalin for subsequent immunohistochemical evaluation.

Immunohistochemistry

Fixed brains were processed for standard paraffin embedding, and 5 µm thin mid-coronal sections were cut on a rotary microtome. Mid-coronal sections were obtained from approximately Bregma -3.00 to 3.60 mm to reveal the thalamic region (Paxinos and Watson 2006). After deparaffinising, sections were heated in a citrate-based antigen unmasking solution to facilitate antigen retrieval, pH 6.0 (Vector®, Burlingame, CA, USA; #H3300), for approximately 30 min in a steamer and cooled on the bench at room temperature for 30 min. Endogenous peroxidase blocking was performed with 0.3% hydrogen peroxide in phosphate-buffered saline for 10 min. Sections were then incubated for 21/2 h at room temperature with primary antibodies diluted in a universal antibody diluent and blocking reagent, Ultra Cruz® Blocking Reagent (Santa Cruz, USA). Cleaved caspase-3 antibody incubations were performed at 1:200 dilution (Cell Signaling Technology, USA; #9661), IBA1 (Cell Signaling, USA; #17198) at 1:1250, Olig2 (ThermoFisher, USA; #13999-1-AP) at 1:1500, MBP (Cell Signaling, USA; #78896) at 1:1500, and Nrf2 (ThermoFisher, USA; #PA1-38312) at 1:100. After washing, sections were incubated in ImmPRESS™ (Peroxidase) Polymer Anti-Rabbit IgG Reagent, made in horse (Vector® #MP-7401). The colour was developed with the DAB Peroxidase (HRP) Substrate Kit (Vector® #SK-4100), and sections were counter-stained in haematoxylin (Ijomone et al. 2018b; Erukainure et al. 2019).

Digital Image Analysis

Immunostained slides were digitised with the Pannoramic 250 Flash II slide scanner (3D Histech, Budapest, Hungary). Nine to twelve random non-overlapping photomicrographic fields (area = 347 x 179 μm^2) of the

thalamic regions were captured at ×400 magnification using the accompanying digital microscopy platform, CaseViewer. Digital images were imported into the National Institutes of Health open imaging analysis software, ImageJ, and analysed using the ImmunoRatio plugin and the cell counter tool. The ImmunoRatio plugin gives a ratio of brown DAB (positive immunoreactivity) and haematoxylin counterstain by digital colure deconvolution. The cell counter tool keeps track of the number of manually selected cell types (Erukainure et al. 2019; Akingbade et al. 2021). The average scores of the photomicrographs analysed were used for data analysis.

Statistical Analysis

Data were analysed using a one-way ANOVA followed by Turkey's multiple comparison tests with GraphPad Prism Version 8 (GraphPad Inc., San Diego, US) statistical software. Further statistical differences between the two groups were confirmed using the Student's t-test where necessary. Statistical significance was set to $P < 0.05$.

RESULTS

Effect of co-exposure to stress with Mn and Ni on thalamic-cleaved caspase-3 levels

Cleaved caspase-3-positive cells showed significant changes across all groups when compared to the control (p <0.01; $F_{(5, 18)} = 35.97$). Turkey's post-hoc test showed a significant ($p < 0.01$) increase in cleaved caspase-3 immunopositivity in all treated groups compared to the control. Furthermore, the Mn-only group showed significantly elevated cleaved caspase-3 immunopositivity compared to the stress-only and other groups (Fig. 1).

Effect of co-exposure to stress with Mn and Ni on thalamic Iba1 levels

Iba1-positive cells showed significant changes across all groups compared to the control (p <0.01; $F_{(5, 18)} = 81.65$). Turkey's post-hoc test showed a significant ($p < 0.01$) decrease in Iba1 immunopositivity in the stress only, Ni, stress+Mn, and stress+Ni groups compared to the control. Contrastingly, the Mn-only group showed significantly ($p < 0.01$) elevated Iba1 immunopositivity compared to the control and other groups (Fig. 2).

Effect of co-exposure to stress with Mn and Ni on thalamic Olig2 levels

Olig2-positive cells showed significant changes in stress-only, Mn, and stress+Mn groups when compared to the control (p <0.01; $F_{(5, 18)} = 16.01$). Turkey's post-hoc test showed a significant ($p < 0.01$) increase in Olig2 immunopositivity in the stress+Mn group when compared to the control and other groups (Fig. 3).

Effect of co-exposure to stress with Mn and Ni on thalamic MBP levels

MBP immunoreactivity showed significant changes across all groups when compared to the control (p <0.01; $F_{(5, 18)} = 10.75$). Turkey's post-hoc test showed a significant ($p < 0.05$) decrease in MBP immunoreactivity across all treated groups compared to the control (Fig. 4).

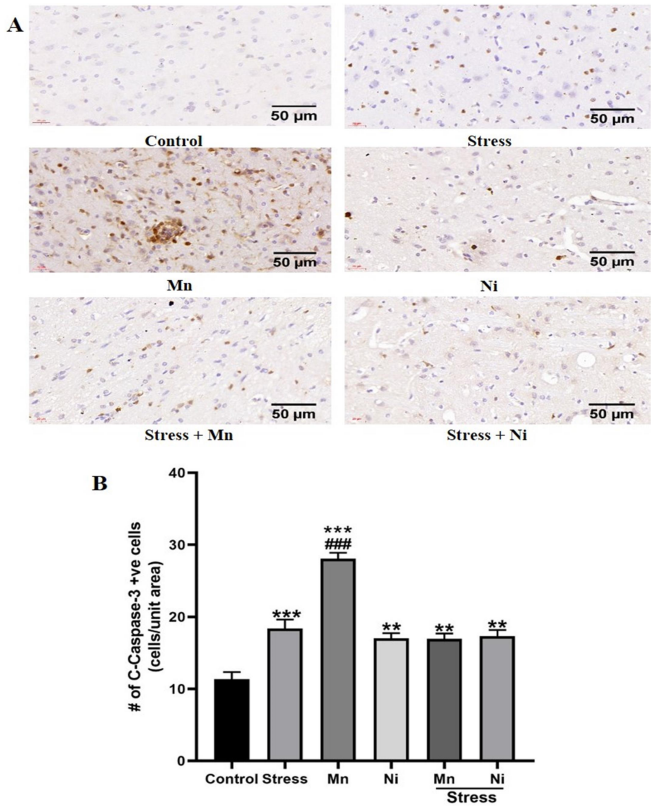

Fig. 1: A. Immunohistochemical demonstration of cleaved caspase-3 in the thalamus of rats exposed to stress, Mn, Ni, Stress+Mn, and Stress+Ni. **B.** Quantification of cleaved caspase-3 immunoreactivity. **p < 0.01, ***p < 0.001 versus the control group; ###p < 0.001 versus stress only group.

Co-exposure to stress with Mn and Ni decreases thalamic Nrf2 immunoreactivity

One-way ANOVA analysis of nrf2 immunoreactivity showed significant changes across all groups when compared to the control (p <0.01; $F_{(5, 18)} = 17.66$). Turkey's post-hoc test showed a significant ($p < 0.05$) decrease in nrf2 immunoreactivity across all treated groups compared to the control (Fig. 5).

DISCUSSION

Excessive accumulation of Mn and Ni has been shown to have neurotoxic impacts in humans and other organisms, resulting in various neurological complications that impair the normal function of the body (Ijomone et al. 2020b; Ijomone 2021). Humans are exposed to varying degrees of stress daily, which, when excessive, could result in various neurological complications (Nash et al. 2019). The thalamus processes and relays sensory information from the external environment to various cortical regions (Georgescu et al. 2020). Thus, this study investigated the

thalamus for capapse-3-mediated apoptosis, microglia response via Iba1 activities, nrf2 regulation of oxidative stress, as well as myelin formation and integrity via Olig2 and MBP activities, respectively, following combined exposures to the chronic stress paradigm and Mn or Ni.

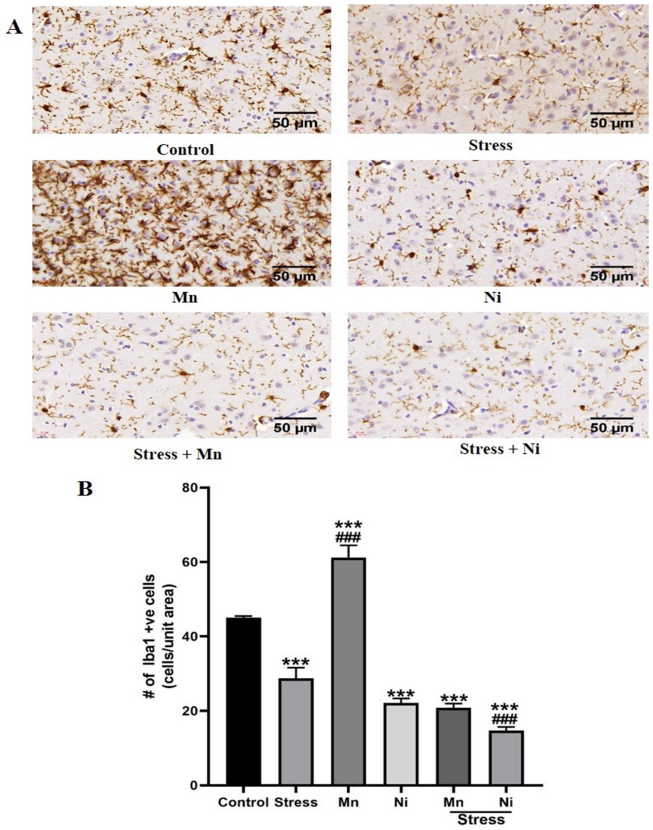

Fig. 2: A. Immunohistochemical demonstration of Iba1 in the thalamus of rats exposed to stress, Mn, Ni, stress + Mn, and stress + Ni. **B.** Quantification of Iba1 immunoreactivity. **p < 0.01, ***p < 0.001 versus the control group, ###p < 0.001 versus stress only group.

The present study observed increased cleaved caspase-3 expression across all experimental groups, though at varying degrees when compared to the control. Chronic restraint stress has previously been demonstrated to trigger the stress response, triggering an increase in caspase-3 and, subsequently, apoptosis in rats (Lehner et al. 2015; Seo et al. 2016). In a study, restraint stress increased the levels of corticosterone in mice (Liang et al. 2013) and cortisol in Cynomolgus monkeys (Shirasaki et al. 2013), resulting in apoptosis. Furthermore, there was an increased and marked elevation of cleaved caspase-3 immunoexpression on Mn exposure. Mn exposure has previously been linked to apoptosis, resulting from elevated caspase-3 expression (Deng et al. 2021). Also, in support of our present results, previous studies have demonstrated that Ni caused increased caspase-3 levels and, consequently, apoptosis in the caecal tonsils of broilers (Wu et al. 2014) and hippocampuses of rats (Ijomone et al. 2018b). However, we surprisingly observed that co-exposures to stress and metals did not exacerbate caspase-3 levels as anticipated.

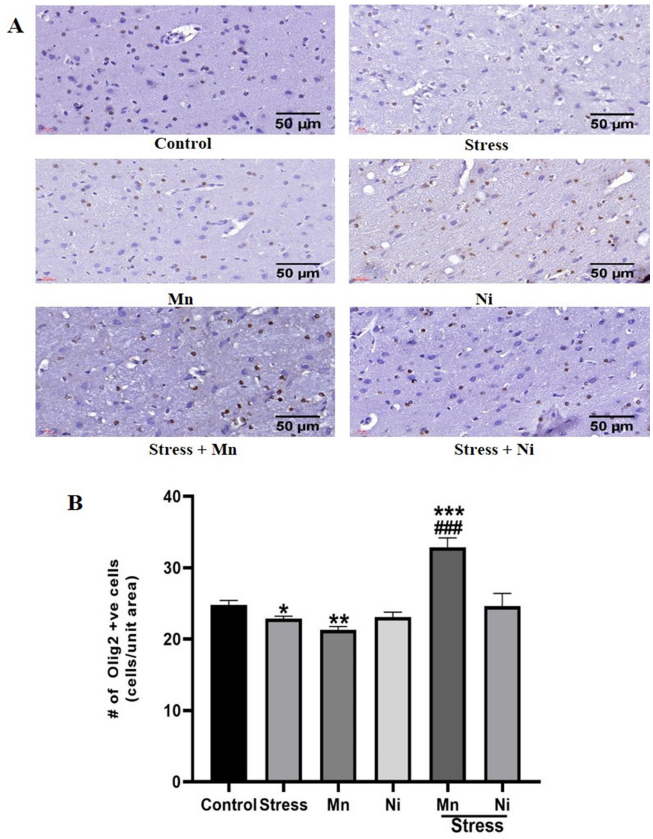

Fig. 3: A. Immunohistochemical demonstration of Olig2 in the thalamus of rats exposed to stress only, Mn, Ni, stress+Mn, and stress+Ni. **B.** Quantification of Olig2 immunoreactivity. *p <0.05 **p < 0.01, ***p < 0.001 versus the control group, ###p < 0.001 versus stress only group.

Microglial cells have been described as the first line of defence in brain pathologies (Li et al. 2020). We employed Iba1 as a marker for microglia activation. A significant increase in Iba1 expression was observed following Mn exposure. Mn exposure has been previously linked to the progression of pathologic activation of microglia neuroinflammatory proteins such as nitric oxide (Tjalkens et al. 2017), leading to neurotoxic damage (Kirkley et al. 2017). In many cases of metal neurotoxicity, increased Iba1 expression is observed and is indicative of microglial activation (Harischandra et al. 2019; Martínez-Hernández et al. 2023). Contrastingly and surprisingly, we observed decreased thalamic Iba1 expression in other treatment groups compared to control. Perhaps there is an underlying mechanism, because of these exposures, that leads to the death and loss of function of the microglial cells. Long-term exposure to stress has been shown to decrease Iba1 mRNA in the dentate gyrus of both rats and mice (Kreisel et al. 2014). Recent reports have also shown that chronic stress induces the loss of microglial cells in the hippocampus (Gong et al. 2018).

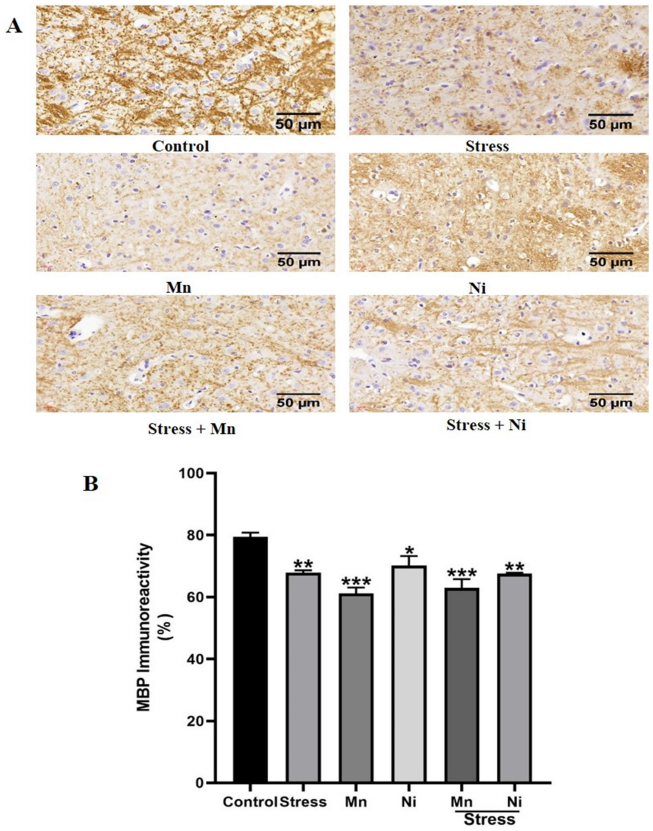

Fig. 4: A. Immunohistochemical demonstration of MBP in the thalamus of rats exposed to stress, Mn, Ni, stress+Mn, and stress+Ni. **B.** Graphical representation of the level of MBP immunoreactivity. *p <0.05 **p < 0.01, ***p <0.001 versus control

There was a reduction in Olig2-positive cells across treatment groups when compared to the control. In the CNS, mature oligodendrocytes are known to generate myelin. However, because of their high metabolic rate and low amounts of antioxidative glutathione, they are particularly susceptible to oxidative damage (Roth and Núñez 2016; Chandley et al. 2022). Exposure to chronic stress in the prefrontal cortex of mice was associated with reduced proliferation of oligodendrocytes (Liu et al. 2018). Therefore, we suggest that exposure to restraint stress reduced oligodendrocyte proliferation in the thalamus and, subsequently, the number of Olig2-expressing cells. The present study showed a decrease in the expression of Olig2 in the Mn and Ni-treated groups. Heavy metals have been shown to cause dysfunctional responses in oligodendrocytes. Therefore, oligodendrocyte precursor cells, which migrate to impaired locations after metal toxicity to support the axon of a damaged neuron, may stop differentiating into mature oligodendrocytes, hence affecting oligodendrocyte regeneration (Maiuolo et al. 2019). Further, Mn has been shown to reduce Olig2 expression levels by inhibiting neural development (Taniguchi et al. 2014). Contrastingly, we observed an increase in Olig2 immunopositivity in the stress+Mn group when compared to the Mn group. As the brain's defence mechanism, we suggest that the increased Olig2 expression occurred as a result of the recruitment of oligodendrocytes to combat demyelination caused by the combined action of Mn and stress (de Castro et al. 2013; Madadi et al. 2019).

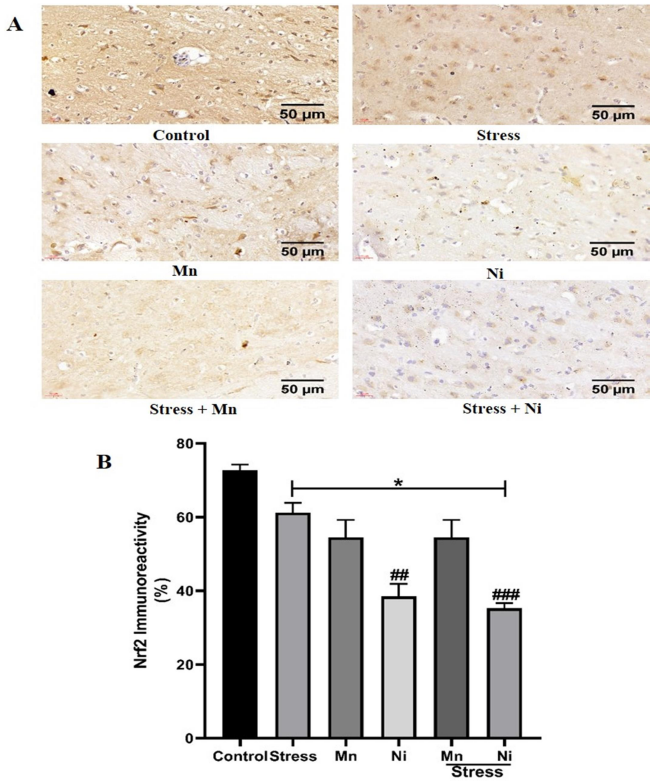

Fig. 5: A. Immunohistochemical demonstration of nrf2 in the thalamus of rats exposed to stress, Mn, Ni, stress+Mn, and stress+Ni. **B.** Graphical representation of the level of nrf2 immunoreactivity. *p <0.05 **p < 0.01 versus control. ##p < 0.01, ###p < 0.001 versus stress only

The results of the present study showed significant reductions in MBP immunoreactivity in both stress and metals treatments. Chronic stress exposures have been previously shown to reduce MBP expression (Luo et al. 2019). Abnormally elevated corticosteroid, which is readily observed during stress, may lead to delayed myelination, consequently impairing CNS development (Miguel-Hidalgo et al. 2019). Similar to our findings, previous reports show that Mn overexposure significantly reduced MBP expression on peripheral nerves (Amos-Kroohs et al. 2019). Also, in rainbow trout, Ni treatment triggered brain damage that was characterised by demyelination and necrotic alterations (Topal et al. 2015).

Result from the present study showed decreases in nrf2 immunoreactivity in both stress, metals, and combined stress with metal treatments, which is contrary to previously reported evidence by (Buha et al. 2021; Ijomone et al. 2022). Insufficient nrf2 activation, which results to decreased nrf2 levels have been shown to occur in certain disease conditions (Gümüş et al. 2022) including Parkinson's and Alzheimer's diseases (Sandberg et al. 2014). A study utilizing Mn and Ni, showed the lack of nrf2

activation preceding cytotoxicity: In their study they suggested that these heavy metals induce cytotoxicity through a mechanism other than oxidative stress (Simmons et al. 2011). However, a more recent study revealed oxidative stress-mediated apoptotic cell death, to be one of the well-established mechanisms of Mn toxicity (Tarale et al. 2016). Another study showed that decreased levels of nrf2 could result from dysfunctional phosphorylation of nrf2 during translocation from the cytoplasm to the nucleus, leading to its breakdown (Sandberg et al. 2014). There was decreased nrf2 immunoreactivity on Mn exposure. In a study by Ijomone et al. (2022), Mn-induced upregulated nrf2 expression in the cortex was observed in female rats only, male rats failed to exhibit similar change despite significant Mn accumulation. The sex-related difference observed in the study was linked to the presence or absence of the hormones, such as oestrogen and β-estradiol. Restraint stress has been shown to decrease antioxidant status of the rat brain (Batandier et al. 2020). However, the result from the present study revealed that stress did not impact the events surrounding nrf2 activation following co-exposure with Mn and Ni.

Conclusion

This study shows that exposure to stressful conditions or metal (Mn and Ni) toxicity triggers apoptosis, microglia dysfunction, myelin disruption, and perturbed oxidative stress regulation in the thalamus. Further, a combination of both stressed conditions and metal (Mn and Ni) exposures exacerbated glia alterations and oxidative stress dysregulation in the thalamus. Although it is worth noting that Mn exposure hyperactivated apoptotic response and microglia activation irrespective of stressful events: Taken together, the study shows that a combination of stressful events and metal exposures, a common occurrence in urban areas, could have an aggravating impact on brain health.

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Conflict of Interest

None declared.

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Authors' Contribution

Conceptualization – OMI; Study design – OMO and OMI; Animal treatment, immunohistochemistry, image acquisition and data analysis – VEA, JFI, OOA, EAA; Manuscript draft – VEA; Critical revisions and editing – OMO and OMI. All authors approved the final draft.

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