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Original Research Article

Memory-enhancing activity of verapamil in murine models of stress

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Abstract

Purpose: *To evaluate the benefit of verapamil on stress-induced memory impairment in mice.*
Methods: *Forty-eight (48) mice were used in this study. They were divided into two equal groups based on the two models of stress used in this study (sleep deprivation and hypoxia). Each model was further divided into 4 groups of six animals each. The mice in the sleep deprivation model were suspended on a platform above water while in the hypoxic model, mice were locked in an airless container for 20 min daily throughout the experiment. As for the intervention, 25 and 50 mg/kg verapamil were pre-administered via the oral route to study groups, except the normal control group and negative control group in both models. After seven-day stress and intervention, the mice were subjected to behavioural tests (Y-maze and object recognition tests), biochemical assays (for acetylcholinesterase activity) and histochemical analysis.*
Results: *Stress caused a significant ($p < 0.05$) impairment in the consolidation and retrieval of working and recognition memories. Also, acetylcholinesterase activity was significantly ($p < 0.05$) enhanced in the stressed groups when compared to control groups. Similarly, the histological analysis revealed a significant decline in population ($p < 0.05$), distribution and density of viable neurons in specific areas of the hippocampus and prefrontal cortex. These alterations were significantly ($p < 0.05$) attenuated in verapamil-treated groups almost in a dose-dependent pattern.*
Conclusion: *Verapamil displays significant memory-enhancing effects in two (2) murine models of stress. Antihypertensives should therefore be considered a viable prospect in the management of stress-related memory disorders after additional studies have been done to establish the mechanisms of action.*

Keywords: *Stress, Sleep deprivation, Hypoxia, Verapamil, Calcium channel blocker, Memory impairment*

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INTRODUCTION

Verapamil is a non-dihydropyridine calcium channel blocker (CCB) of the phenylalkylamine class whose usefulness in the cardiovascular system has existed for almost 60 years. Prior to this time, verapamil (VPM) has been indicated for the management of hypertension, angina

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pectoris and arrhythmia [1]. Even though the primary role of CCBs is lowering vascular pressure, some studies have dedicated time to exploring their secondary roles [2]. This has made it possible for verapamil, as well as other CCBs, to be considered in the management of several diseases/disorders such as cluster headaches, bipolar disorder, diabetes, cancer, epilepsy, and neurodegenerative diseases such as Alzheimer's disease (AD) [3]. Alzheimer's disease is a neurodegenerative disease whose key feature is memory loss and cognitive decline. In some studies, treatment with antihypertensive drugs (especially CCBs) caused a significant improvement in memory and cognition in addition to their antihypertensive effect [4]. This may be because both diseases (hypertension and AD) have roots in inflammation. However, it is imperative to note that the risk of developing AD increases significantly with the comorbidity of cardiovascular diseases such as hypertension [5].

Furthermore, Marco-Contelles and colleagues once created a hybrid ligand called the *tacripyrines* by coupling a dihydropyridine called *nimodipine* with an AD drug called *tacrine*. This hybrid demonstrated superb neuroprotection in addition to its calcium channel-blocking ability and emerged to be a lead candidate in the treatment of AD [6]. In another study, VPM counteracted mitochondrial damage in the brain by causing a significant (i) decrease in the activity of prooxidant enzymes, (ii) increase in the activity of antioxidant enzymes, and (iii) decrease in the activity of apoptotic factors [7]. Besides these benefits, CCBs also possess antitau, antiamyloid, antiplatelet, anti-phospholipase, antioxidant activity, neuroprotective activity and anti-inflammatory effects [4]. Since these phenomena usually precede the onset of AD, it is safe to suggest that CCBs can be used to counteract cognitive decline and memory impairment. From the above, the authors observed a paucity of information on the role of CCBs on memory impairment induced by external stress or stressful conditions even though calcium signaling is implicated in many human processes including memory formation. As a result, this current study was designed to investigate the memory-enhancing effect of verapamil on mice exposed to two pharmacological models of stress.

EXPERIMENTAL

Mice

Fifty (50) Swiss albino mice (body weight: 22.0 – 26.0 g) used in the study were obtained from the Central Animal House, College of Health Sciences, Delta State University, Abraka. They were housed in transparent cages and allowed to acclimatize in the laboratory at ambient temperature and humidity under 12 hourly light/dark conditions. Food and water were also provided *ad libitum*. The study was performed in conformity with the Guide for the Care and Use of Laboratory Animals of the National Research Council [8] and ethical approval was granted by the Ethics Committee of Delta State University, Abraka (approval no. FBMS/DELSU/21/105).

Drug information

Mass volumes of verapamil (VPM) (25 and 50 mg/kg) were prepared in the two doses required by serial dilution in water. The doses were selected based on results from pilot studies.

Hypoxia-induced stress (HS) paradigm

Twenty-four (24) mice were exposed to hypoxia by intermittently locking each mouse in a small airtight vessel as described by Adebayo *et al* [9]. The mice were allocated into four groups of six mice each (i.e. n = 6) and treated *per os*:

- Group 1 (control) received vehicle only, i.e., distilled water (VEH 10 mL/kg)
- Group 2 (stressed) received distilled water and was subsequently exposed to hypoxia (VEH 10 mL/kg + HS)
- Group 3 received verapamil and was subsequently exposed to hypoxia (VPM 25 mg/kg + HS)
- Group 4 received verapamil and were subsequently exposed to hypoxia (VPM 50 mg/kg + HS)

The mice in groups 2 - 4 were subjected to the hypoxia protocol approximately one hour after the administration of distilled water or verapamil, respectively, daily. Each mouse was locked in for 20 min daily for seven consecutive days. On day 8, each mouse was subjected to Y-maze, and novel object recognition tests, a biochemical analysis of acetylcholinesterase activity as well as histology of specific brain regions.

Platform-over-water model of sleep deprivation (SD)

Deprivation of the rapid eye movement (REM) phase of sleep was the target of this protocol as developed by Eduviere *et al* [10]. It is based on the principle that the mouse will fall off the

platform and into the water at the outset of the REM phase of sleep owing to the muscle relaxation that characterizes this phase. Twenty-four (24) mice were individually placed on a wooden platform with grids suspended inside a plastic cage containing water.

The grids were approximately 2 cm apart, the cage was filled with water to ~1 cm below the platform surface and rodent pellets were placed on the platforms above the water f throughout the study. The mice were divided into four groups of six mice each (i.e. n = 6) and treated *per os*:

- Group 1 (control) received vehicle only (VEH 10 mL/kg)
- Group 2 (stressed) received distilled water and were sleep-deprived (VEH 10 mL/kg + SD)
- Group 3 received verapamil and was sleep-deprived (VPM 25 mg/kg + SD)
- Group 4 received verapamil and was sleep-deprived (VPM 50 mg/kg + SD)

All animals were treated daily for seven (7) consecutive days while the mice in groups 2-4 were subjected to this protocol daily for the final 72 h. Soon after the 72nd h, each mouse was subjected to Y-maze, and novel object recognition tests, a biochemical analysis of acetylcholinesterase activity as well as histology of specific brain regions.

Behavioural tests

Y-maze test

As the name implies, the Y-maze apparatus is a wooden or metal *Y* with each arm compartment at 120° from the other. The test was carried out to ascertain the spatial alternation performance of each mouse. This is considered an index of memory where the ability of the mouse to recall its previous location on the *Y* is tested.

Since each arm is labeled, the mice were placed in the same arm to begin exploring for 5 min. An alternation was defined as a complete entry into the three arms in a consecutive order. Then, the percentage alternation, which is a measure of spatial working memory, was determined.

Novel object recognition test (NORT)

Three identical objects (usually bottles) are labeled A, B, and C whereas object C is coloured differently from objects A and B. In the trial phase, each mouse is placed in the open field chamber with objects A and B for 5 min. Afterward, the mouse is returned to its home cage for 2 h before the test phase begins. In the test phase, object C is used to replace object B and the mouse is allowed to explore for 5 min. The time spent exploring the familiar object and the novel object were recorded separately and used to calculate the percentage preference which is an index of recognition memory.

Collection of brain samples

A day after behavioural tests, thirty-two (32) mice were sacrificed by decapitation (that is, 4 mice from each group and 16 mice from each model). Soon after, whole brains were harvested from all of them and first fixed in 10 % phosphate-buffered formalin in preparation for biochemical assays.

Determination of acetylcholinesterase (AChE) activity

To separate the supernatant, the 32 harvested brains were homogenized using a phosphate buffer and cold centrifuged for 15 min at 10,000 rpm. Acetylcholinesterase (AChE) activity was determined colorimetrically using the AChE activity assay kit from Sigma-Aldrich® USA. The assay protocol uses Ellman's reagent and measures the AChE activity using a spectrophotometer and expresses it as μmol/min/g tissue.

Histology and assessment of neuronal density

The remaining sixteen (16) unsacrificed mice (2 from each group and 8 from each model) were subjected to intracardiac perfusion using 10% phosphate-buffered formalin in order to retain cellular and vascular integrity after death. Afterward, the brains were harvested and fixed in the same 10% phosphate-buffered formalin.

Following the procurement of the whole brains, manual sectioning of each brain was carried out and tissue slices of the hippocampus (cornuammonis 1 and 3; CA1 and CA3) and prefrontal cortex (PFC) were obtained using a microtome and processed by the routine method for paraffin wax embedment in preparation for Hematoxylin and Eosin (H & E) staining [11]. Counting of viable neuronal cells was done with the Image J software. Neuronal density was thus calculated as a ratio of viable neuronal cell counts to square area of the circular view in a section.

Statistical analysis

The Graph Pad® software version 8 was used for analysis. Data obtained are presented as mean ± Standard deviation (SD). The results were analyzed by one-way ANOVA, followed by Tukey's range posthoc test. Significance level was set at $p < 0.05$.

RESULTS

Effect of verapamil on working memory in stressed mice

Working memory was assessed in mice by subjecting them to the Y-maze test. It was observed that stress (sleep deprivation and hypoxia) caused impairment in working memory formation and retention as shown by the low percentage alternation recorded in the Y-maze test. Also, the sleep-deprived mice as well as the hypoxic mice demonstrated a significantly ($p < 0.05$) lower percentage alternation than the control groups in both tests. However, the verapamil-treated groups showed a significant ($p < 0.05$) increase in working memory when compared with the stressed groups in both models (Figure 1).

Effect of verapamil on recognition memory in stressed mice

Recognition memory was assessed in mice by subjecting them to the NORT. It was observed that stress (sleep deprivation and hypoxia) caused impairment in formation and retention of recognition memory as shown by the low recognition index recorded in the NORT. Also, the sleep-deprived mice as well as the hypoxic mice (Figure 2) exhibited a significantly ($p < 0.05$) lower recognition index than the control groups in the test. However, the verapamil groups showed a significant ($p < 0.05$) increase in recognition memory when compared with the stressed groups.

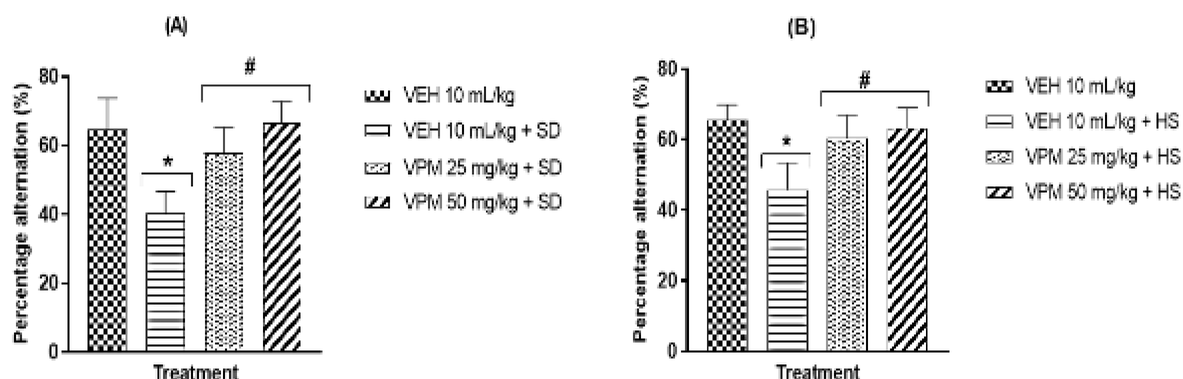

Figure 1: Verapamil repaired working memory in sleep deprivation-induced stress (A) and hypoxic stress (B). ***Note***: *$P < 0.05$ vs control group; #$p < 0.05$ vs stressed group, VEH, control group; VEH + SD, stressed group; VEH + HS, stressed group; VPM + SD, verapamil treated stressed groups

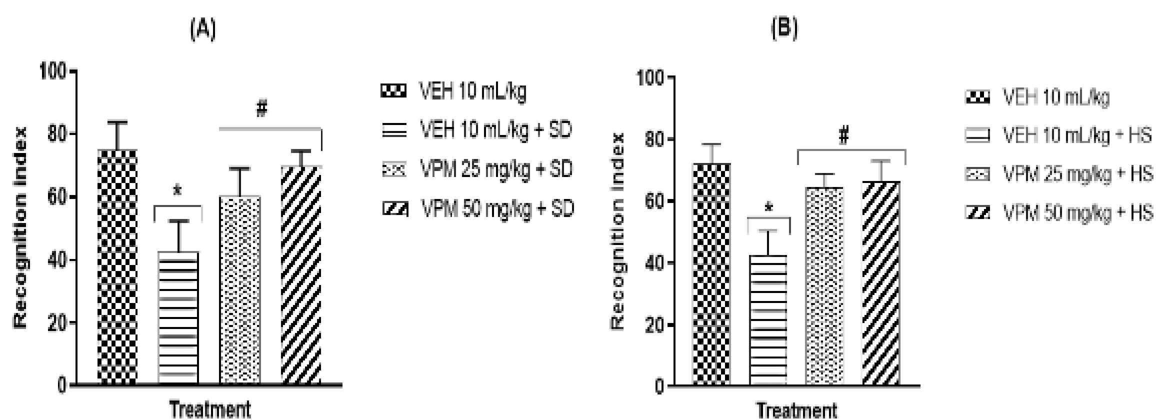

Figure 2: Verapamil repaired recognition memory in sleep deprivation-induced stress (A) and hypoxic stress (B) **Note:** *$p < 0.05$ vs control group; #$p < 0.05$ vs stressed group, VEH, control group; VEH + SD, stressed group; VEH + HS, stressed group; VPM + SD, verapamil treated stressed groups

Effect of verapamil on acetylcholinesterase activity in stressed mice

The test for its activity in this study revealed a significant ($p < 0.05$) increase in the sleep-deprived (Figure 3 A) and hypoxic (Figure 3 B) groups when compared to the control groups. Also, verapamil administration seemed to significantly ($p < 0.05$) inhibit AChE activity when compared to the stressed groups.

Effect of verapamil on hippocampal CA1 neuronal distribution and density in stressed mice

As shown in the photomicrographs presented in Figure 4 below, a significant alteration in the distribution of cells of the hippocampal cornuammonis 1 (CA1) region was identified in the stressed group of both models (slide SD and HS) compared to their control groups (slide NC). This alteration was however corrected significantly by verapamil in both protocols (slides V25 and V50). In the same vein, the decline in neuronal density observed in the stressed groups of both models was attenuated significantly ($p < 0.05$) by verapamil (Figure 5).

Effect of verapamil on hippocampal CA3 neuronal distribution and density in stressed mice

As shown in the photomicrographs presented in Figure 6 below, a significant alteration in the distribution of cells of the hippocampal cornuammonis 3 (CA3) region was identified in the stressed group of both models (slide SD and HS) compared to their control groups (slide NC). This alteration was however corrected significantly by verapamil in both protocols (slides V25 and V50). In the same vein, the decline in neuronal density observed in the stressed groups of both models was attenuated significantly ($p < 0.05$) by verapamil (Figure 7).

Effect of verapamil on distribution and density of prefrontal cortex neurons in stressed mice

As seen in Figure 8 below, a significant scantiness in the distribution of cells of the prefrontal cortex (PFC) was observed in the stressed group of both models (slide SD and slide HS). More particularly, the viability of the vacuolated and pyknotic cells of the hypoxic group (slide HS) was affected when compared to the normal control group (slide NC). However, this scantiness was significantly resolved by verapamil (slides V25 and V50). In the same vein, the decline in the density of PFC neurons observed in the stressed groups of both models was attenuated significantly ($p < 0.05$) by verapamil (Figure 9).

DISCUSSION

This study evaluated the effect of a known antihypertensive on memory dysfunction and possible cognitive decline induced by sleep and oxygen deprivation in mice.

Sleep and oxygen are very fundamental necessities for basic human functioning. Although the key function of sleep is yet to be identified, it is considered a state for cellular regeneration which can only happen in the presence of oxygen. Following from this, we can infer that even a slight disruption in the circadian rhythm and oxygen supply poses a threat to various human processes, central and peripheral alike.

As a matter of fact, the brain is mostly affected by a deprivation of either because it is the organ which consumes the greatest percentage of oxygen in the body [12]. Therefore, sleep deprivation (SD), whether partial or total, and oxygen deprivation otherwise known as hypoxia, are powerful tools for the induction of physical and mental stress.

In this study, working memory was evaluated using the Y-maze, a recognized pharmacological tool for assessing memory. Memory formation and consolidation were impaired in the stressed groups. Stressed groups refer to the mice which were subjected to sleep deprivation and hypoxia without an intervention administered. Stressed groups exhibited a significantly lower percentage alternation between the arms of maze relative to normal control group. Similarly, recognition memory which was evaluated in NORT revealed that stressed groups had a significantly lower preference for novel objects relative to normal control group.

However, treatment with verapamil at both doses enhanced memory function which was evident in the observed significant increase in percentage alternations in the Y-maze and increased preference for the novel object in the NORT when compared with the stressed groups. This agrees with existing literature which found a link between stress and memory dysfunction [13] as well as improvement in memory after verapamil administration [14,15].

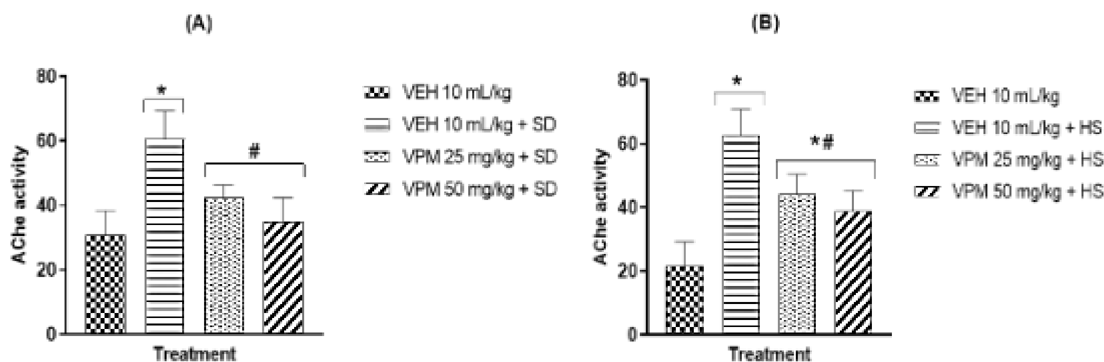

Figure 3: Verapamil inhibited acetylcholinesterase activity in sleep deprivation-induced stress (A) and hypoxic stress (B). ***Note*:** *P < 0.05 vs control group; #p < 0.05 vs stressed group; VEH, control group; VEH + SD, stressed group; VEH + HS, stressed group; VPM + HS, verapamil groups

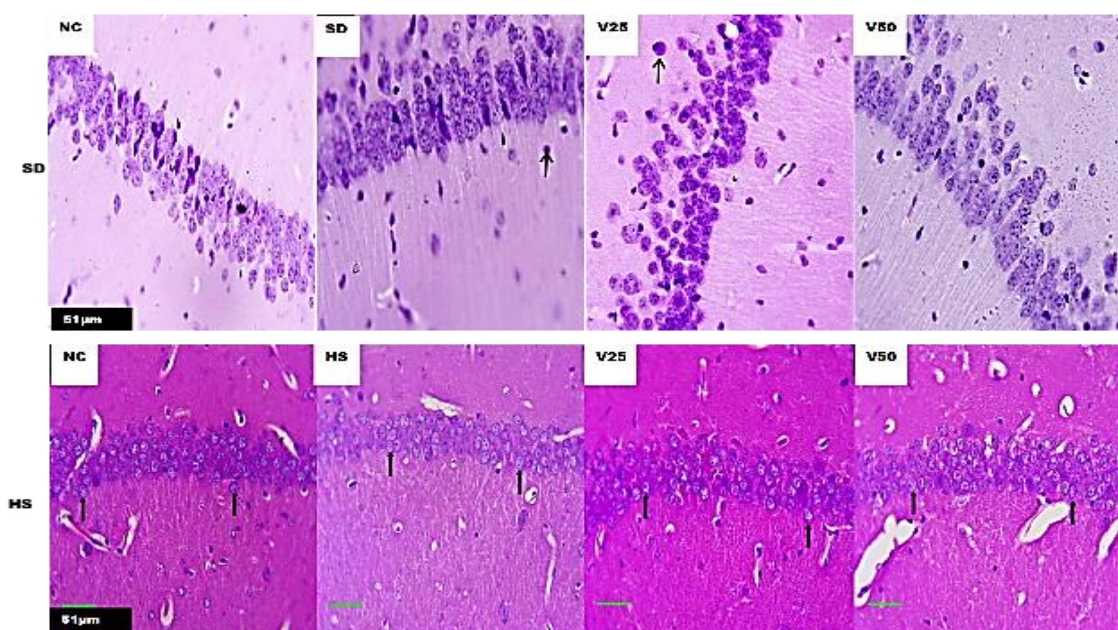

Figure 4: Verapamil evened out the distribution of hippocampal CA1 neuronal cells in sleep deprivation-induced stress (SD) and hypoxic stress (HS). **Note:** Magnification, x400; Scale bar, 51 µm; Black arrows, neuronal cells; NC, normal control; SD, sleep deprivation; HS, hypoxic stress; V25, verapamil 25 mg/kg; V50, verapamil 50 mg/kg

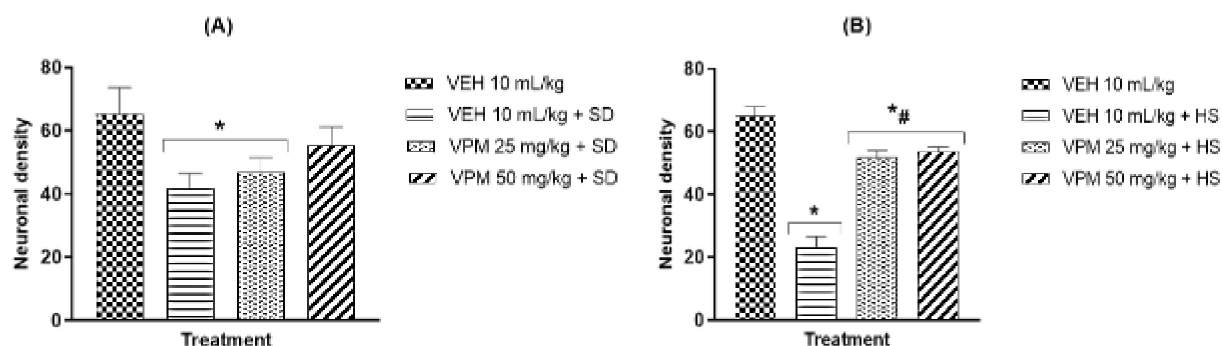

Figure 5: Verapamil enhanced density of CA1 neurons in sleep deprivation-induced stress (A) and hypoxic stress (B). ***Note:*** *P < 0.05 vs control group; #p < 0.05 vs stressed group, VEH, control group; VEH + SD, stressed group; VEH + HS, stressed group; VPM + HS, verapamil treated stressed groups

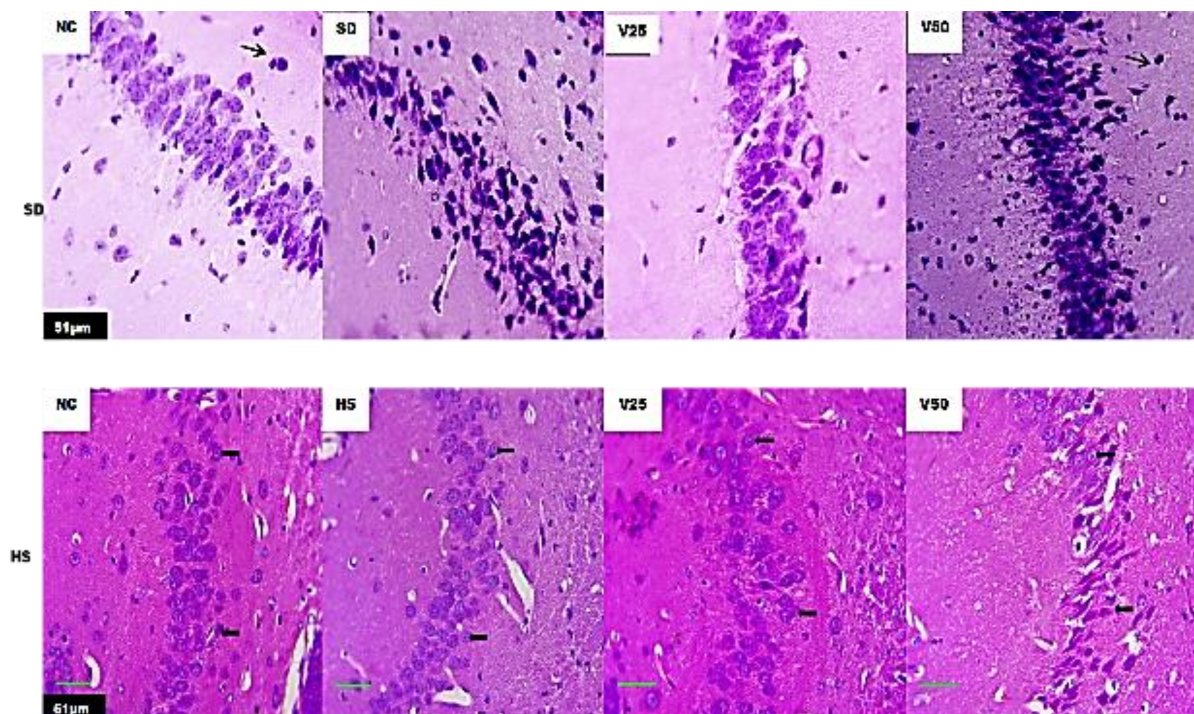

Figure 6: Verapamil restored the distribution of hippocampal CA3 neuronal cells in sleep deprivation-induced stress (SD) and hypoxic stress (HS) **Note:** Magnification, x400; Scale bar, 51 μm; Black arrows, neuronal cells; NC, normal control; SD, sleep deprivation; HS, hypoxic stress; V25, verapamil 25 mg/kg; V50, verapamil 50 mg/kg

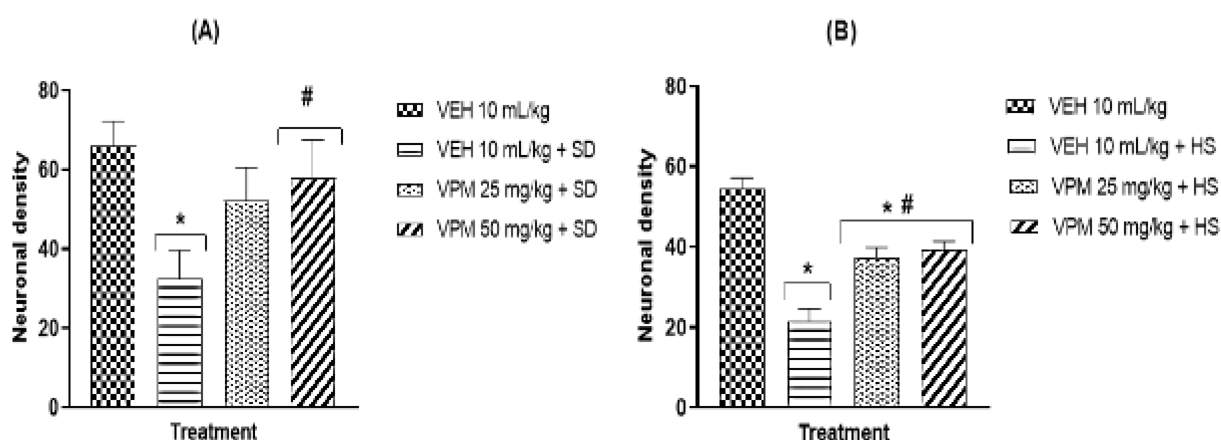

Figure 7: Verapamil enhanced density of CA3 neurons in sleep deprivation-induced stress (A) and hypoxic stress (B) in mice. **Note:** **P* < 0.05 vs control group; #*p* < 0.05 vs stressed group; VEH, control group; VEH + SD, stressed group; VEH + HS, stressed group; VPM + HS, verapamil treated stressed groups

Another recognized determinant of memory function is acetylcholinesterase (AChE) activity. AChE is an enzyme that breaks down acetylcholine thereby preventing overstimulation of nerves, muscles and even glands resulting from excess acetylcholine. Acetylcholinesterase activity was significantly ($p < 0.05$) increased in the stressed groups when compared with normal control groups. This aligns with other studies which showed that AChE activity was increased after exposure to hypobaric hypoxia [16] and sleep deprivation [17]. According to the preceding study, this interference with the cholinergic system leads to a reduction in acetylcholine levels thus impeding neurogenesis which can further result in the disruption of memory formation and consolidation. However, verapamil repaired this breakdown in memory formation by inhibiting AChE activity thus making sufficient acetylcholine available for neurogenesis [14].

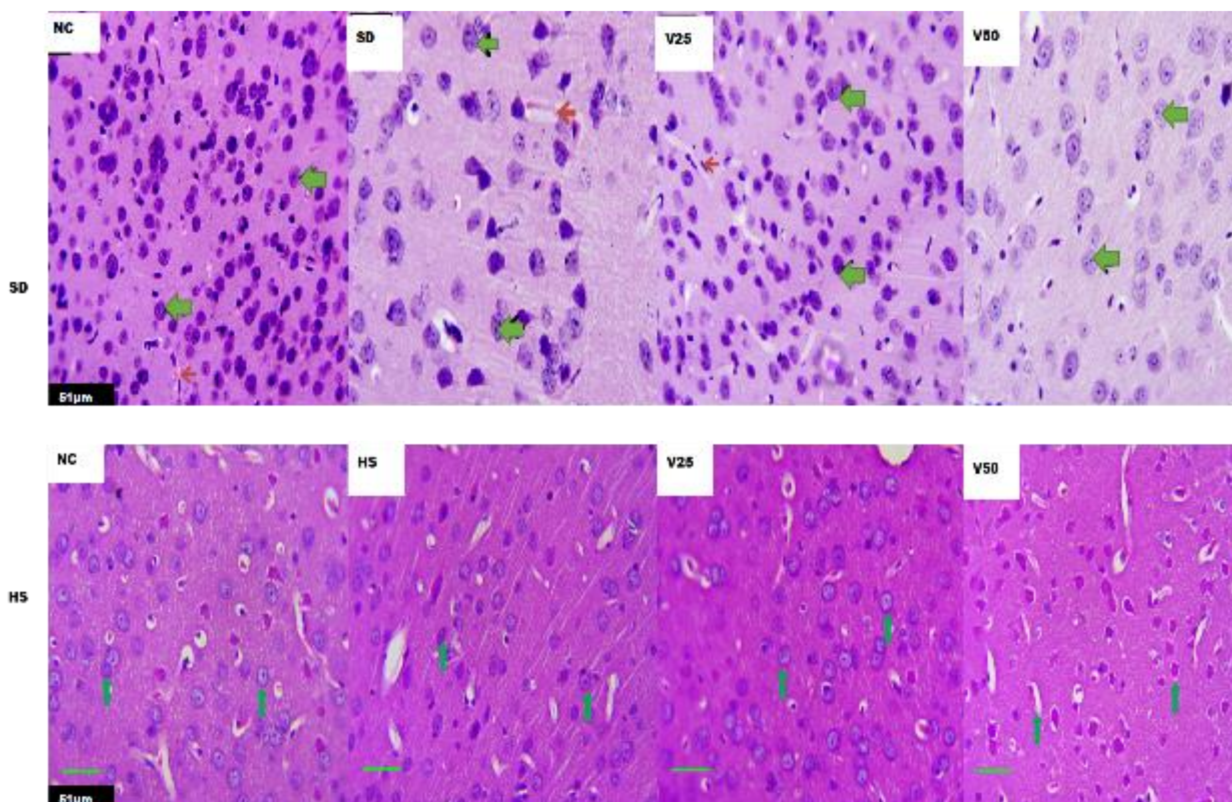

Figure 8: Verapamil restored the distribution and viability of prefrontal neuronal cells in sleep deprivation-induced stress (SD) and hypoxic stress (HS) mice. ***Note:*** Magnification, x400; Scale bar, 51 µm; red arrows, blood vessel; Green arrows, neuronal cells; NC, normal control; SD, sleep deprivation; HS, hypoxic stress; V25, verapamil 25 mg/kg; V50, verapamil 50 mg/kg

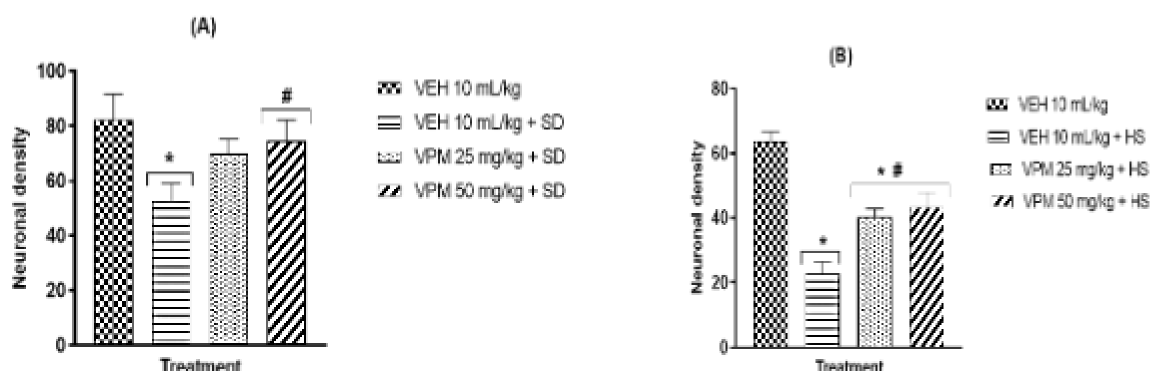

Figure 9: Verapamil enhanced density of prefrontal neurons in sleep deprivation-induced stress in mice. (A) and hypoxic stress (B) mice. **Note**: $^*P < 0.05$ vs control group; $^{\#}p < 0.05$ vs stressed group; VEH, control group; VEH + SD, stressed group; VEH + HS, stressed group; VPM + HS, verapamil treated stressed groups

Furthermore, histological analysis of specific brain regions was carried out in order to assess the extent of damage and restoration done by the stressors and verapamil at the cellular level, respectively. Histology of CA1 and CA3 sections of the hippocampus revealed that stress (i.e., sleep deprivation and hypoxia) caused a significant alteration in the volume and distribution pattern of neuronal cells. Both regions had significantly fewer neurons when compared with the normal control groups. The same was observed in the micrographs of the prefrontal cortex (PFC). This proves consistency with existing studies which agree that stress is neurotoxic [18]. More recent studies have shown that stress alters hippocampal coding [19] and increases the risk of psychological disorders which can be linked to the prefrontal cortex [20].

Limitations of this study

The duration of this study is too short to validate the claim that stress resulting from oxygen or sleep deprivation can trigger pathophysiological

damage that can be reversed by verapamil. It is therefore recommended that long-term exposure to such stressors and a repeated evaluation of specific behaviours in stressed animals.

CONCLUSION

Verapamil displays significant memory-enhancing effects in two (2) murine models of stress. Antihypertensives may therefore be a viable prospect in the management of stress-related memory disorders, but additional studies are required to establish its mechanisms of action. Further studies are also required to understand the complete role of antihypertensives in neurodegenerative disorders, especially those in the calcium-channel blocker class.

DECLARATIONS

Acknowledgement

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Ethical approval

Ethical approval was granted by the Ethics Committee of Delta State University, Abraka. (FBMS/DELSU/21/105).

Use of Artificial Intelligence

The authors declare that artificial intelligence was not used in the writing of this research article.

Use of research reporting tool

The authors declare that no research reporting tools were employed in this research.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Study conceptualization and design was done by Anthony T Eduviere. All authors were involved in the study. Material preparation and data collection were performed by Lily O Otomewo. Analysis was performed by Onoriode A Udi, Adefunke O Opajobi and Emuesiri G Moke. Manuscript writing and review were done by Anthony T Eduviere and Lily O Otomewo. All authors read and approved the final manuscript for publication.

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