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AMELIORATIVE EFFECT OF VITAMIN C AND UV-B RAYS ON NIGROSTRIATAL AND CORTICOSTRIATAL DEGENERATION IN HALOPERIDOL INDUCED PARKINSONISM IN WISTAR RATS

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ABSTRACT

Prolonged inhibition of dopamine-2 receptor (D2R) is shown to cause degeneration of dopamine neurons leading to parkinsonism. Previously we have shown that vitamin D3 receptor stimulation improved motor–cognitive functions in dopamine-2 receptor (D_2R) parkinsonian mice model. Presently, we examined the ameliorative effect of vitamin C and UV-B rays on nigrostriatal and corticostriatal degeneration in drug induced parkinsonism in Wistar rats. Twenty male Wistar rats with average weight of 120 g were distributed into four groups (NS, -D2, -D2+UV-B and –D2+Vit.C). Parkinsonism was induced by administering 10 mg/kg b.wt. of haloperidol for 14 days (intraperitoneally) without and with treatment of 125 mg/kg b.wt. of vitamin C or 2 hours of exposure to morning sunlight between 8-10 am. The animals were subjected to cylinder, pole and stepping test for motor functions. Motor cortex (M1), substantia nigra pars compacta (SNc) and striatum (CPu) were processed and stained using haematoxylin and eosin and Cresyl violet stains. Cell count was done using ImageJ software (version 5). Data were presented as mean ± standard error of mean; analysed using one-way analysis of variance and Tukey's multiple comparison test, and significant level was determined at 0.05 ($p < 0.05$*). Haloperidol induced parkinsonism caused significant bradykinesia (*$p < 0.05$), rigidity (** $p < 0.01$), neuron lost (**$p < 0.01$) and expressions of degeneration hallmarks in SNc and M1. UV-B or Vit. C treatment showed ameliorative potentials in reducing motor deficit experience in parkinsonism, but not regenerating the already lost neurons.

Key words: *Parkinsonism, Vitamin C, UV-B rays, Dopamine-2 receptor blocker, Substantia nigra (SN), Striatum (CPu), Motor cortex (M1)*

INTRODUCTION

Several studies have shown that prolonged inhibition of dopamine-2 receptor (-D2R) causes loss of dopamine neurons, resulting in to parkinsonism (Shirayama et al. 2000; Iderberg et al. 2015). These dopamine neurons project from substantia nigra to striatum (nigrostriatal tract), and are also found in the cortex (corticostriatal tract) (Singh 2009). It is known that haloperidol has the ability to centrally block dopamine-2 receptors (Seeman and Tallerico 2003;

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Karl et al. 2006; Graff-Guerrero et al. 2009). Parkinsonism occurs mostly in aged-population (Dawson and Dawson 2003). However, several cases of juvenile and young-onset of parkinsonism have been reported (Muthane et al. 1994; Schrag et al. 1998; Paviour et al. 2004; Silver et al. 2004). Parkin mutations were found to be frequent in patients with isolated early-onset parkinsonism (Periquet et al. 2003). Drug induced parkinsonism was found be static, i.e it neither progress nor ameliorate upon withdrawal. (Meltzer et al. 2003).

In addition to modulation of dopaminergic neurotransmission systems (Angelow et al. 2003), and increasing the expression of brain-derived neurotrophic factor (Grant et al. 2005), Intraperitoneal ascorbate (125 mg /kg) was found to reversed motor and memory deficits in mice (Calero et al. 2011). This suggests that vitamin C could be used to at least manage prolonged inhibition of dopamine-2 receptor (D_2R) model of parkinsonism.

Our previous studies have shown that vitamin D3 receptor stimulation rescues nigrostriatal neural activity and improves motor and cognitive functions in D_2R parkinsonian mouse model (Ishola et al. 2015) and in Tardive Dyskinesia mouse model (Bankole et al. 2015). Ultraviolet-B rays have the same effect of simulating vitamin D3 receptor as it induces vitamin D production (Rathish and Arun, 2012). Therefore, it is possible that UV-B rays can at least have the same or even better effect of reversing parkinsonism in D_2R model. These two hypotheses called for the research on the role of vitamin C or UV-B rays as a therapy to rescue neural damage induced by dopamine-2 receptor inhibition model of parkinsonism.

MATERIALS AND METHODS

Ethical Clearance

Approval for the study was obtained from the Ethical Committee of the Department of Anatomy, Faculty of Basic Health Sciences, Bayero University, Kano.

Drug Procurement

Haloperidol and vit. C were produced by Swiss Pharma Pvt. Ltd. Gujarat and re-suspended in normal saline. Haloperidol and vit. C solutions were prepared weekly as needed and stored at 4ºC. The animals were exposed to solar UV-B rays for 2 hours between 8 -10 am for UV-B vitamin D3 induction.

Animal Treatment

Twenty male Wistar rats with average weight of 120 g were procured from Biological Sciences department animal house, Bayero University Kano and assigned into 4 groups using simple random sampling as in Table 1. All the groups were left untreated for 7 days of acclimatization.

Behavioural Tests

At the end of the treatment phase, motor coordination of the animals was examined using cylinder, pole and stepping tests. All animals were familiarized with the behavioural test room 72 hours before the commencement of the tests.

Cylinder test

Cylinder test was designed to assess motor coordination asymmetry and deficit in the experimental animals. Each animal was placed in 15 cm in diameter transparent rubber container with an open top and was allowed to explore the walls of the container with the forelimbs while standing on the two hind limbs. Subsequently, the number of times the animal places its forelimb against the wall of the container was taken and recorded (Ishola et al. 2014).

Stepping test

Stepping test was designed to detect speed of motor activities which could be normal, bradykinasia or

Table 1: Animal Treatment

Group	Dose
NS (n = 5)	Animals were administered 0.1 mL (intraperitoneal) of 0.9% normal saline for 21 days (Ogundele et al. 2014).
D_2 (n = 5)	Animals were administered 0.1 mL (intraperitoneal) of 0.9% normal saline for 7 days followed by 10 mg/0.8 mL/kg BW of haloperidol (intraperitoneal) for 14 days (Ogundele et al. 2014).
D_2+Vit.C (n = 5)	Animals were administered 10 mg/0.8 mL/kg BW of haloperidol (intraperitoneal) for 14 days followed by 125 mg/kg BW body weight of Vitamin C (intraperitoneal) for 7 days (Calero et al. 2011; Ogundele et al. 2014).
D_2 + UV-B (n = 5)	Animals were administered 10 mg/kg BW of haloperidol (intraperitoneal) for 14 days followed by UV-B rays exposure of 2 hours daily for 7 daily UV-B rays (Ishola et al. 2015).

akinesia. Both bradykinesia and akinesia have been attributed to be disorder of the Basal ganglia (Berardelli et al. 2001). The Stepping test was conducted on a wooden ramp with length of 1meter connected to the rat cage. The rats were gently placed on the wooden ramp with their heads toward the direction of their cages. The time taken for each rat to initiate stepping by each limb was taken and recorded (Olsson et al. 1995).

Pole Test

Pole test was designed to assess rigidity. The Pole test was conducted on a smooth wooden pole of 1 m in length and 3 cm in diameter fixed in the middle of an empty cage. The rats were placed facing downward at the top of a wooden pole. The time taken for the Wistar rats to move down the Pole was taken and recorded.

Animal Sacrifice

After the behavioural protocol, animals were anaesthetized using 10 mg/kg ketamine. Subsequently, the animals were perfused (transcardially) with normal saline to flush the blood followed by 10% formal saline to preserve the whole animal. Later on, the skull was opened to harvest the whole brain following which it was post-fixed in 10% formalin.

Neural Tissue Procession

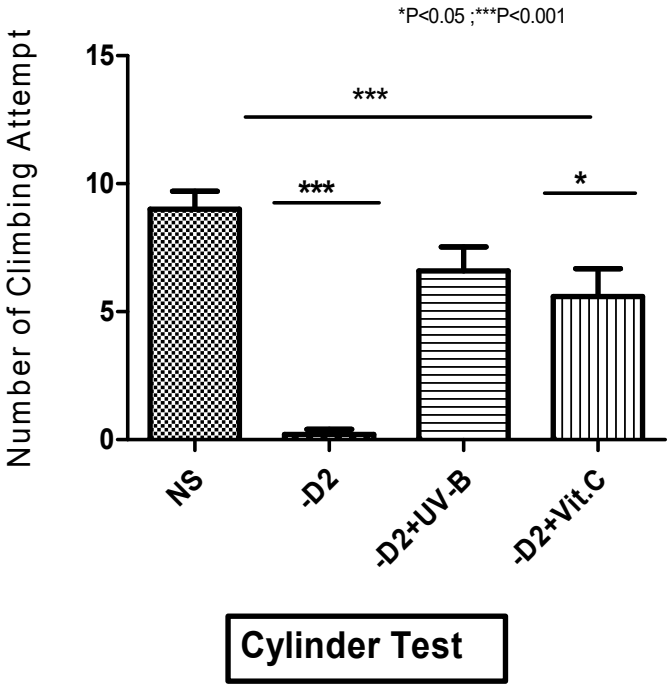

Figure 1: Cylinder Motor Coordination Test for Haloperidol Induced Parkinsonism (HIP), UV-B Treated HIP and Vit. C Treated HIP. *,***Significantly different compared with the control at p<0.05 and 0.001, respectively). Both UV-B and Vit.C groups (-D2+UV-B and –D2+Vit.C) recorded an increase in climbing attempts when compared with the –D2 treatment. s = second

After fixation, the whole brain was dissected on a stereotaxic grid to expose the approximate primary motor cortex (M1), striatum (CPu) and substantia nigra (SNc) to capture corticostriatal and nigrostriatal neurons using a calibrated frame and grid. The dissected brain tissues were processed and paraffin wax embedded tissue blocks were obtained for histology. 5 µm sections were obtained using microtome and were stained routinely in haematoxylin and eosin stains (H & E) and cresyl violet (Nissl stain) which were later evaluated for assessment of neural damage especially degenerative changes in cell body (perikaryon).

Nissl Body Count

Nissl bodies in SNc, CPu and M1 were counted using ImageJ software version 5, after using the appropriate threshold to depict the degenerating neurons, following the method of Peiying (2012).

Statistical Analysis

Data were presented as mean ± standard error of mean; analysed using one-way analysis of variance and Tukey's multiple comparison test. Statistical significance was set as $p < 0.05$*

RESULTS

Motor Function Tests

After inducing parkinsonism with haloperidol, the motor activities of the parkinsonian rat models both treated groups (-D2+UV-B and –D2+Vit.C) and untreated (-D2) were compared with the control group (NS) and one another in cylinder, pole and stepping tests.

Cylinder Test

Haloperidol induced parkinsonism (-D2) (0.20 ± 0.20) caused significant ($p < 0.001$) reduction of climbing attempts when compared with the NS (9.0 ± 0.70). Vitamin C treated parkinsonism (-D2+Vit.C) (5.60 ± 1.07) shows less significant ($p < 0.05$) reduction of climbing attempt when compared with NS.

There was no significant change in ultraviolet B rays treated parkinsonism (-D2+UV-B) (6.6 ± 0.93) when compared with the control (NS). However, there was highly significant improvement in the climbing attempt of -D2+UV-B when compared with –D2 ($p < 0.001$). A significant improvement in climbing attempt was also seen in –D2+Vit.C when compared to –D2 ($p < 0.01$). There was no significant difference between both treatment groups (–D2+UV-B and –D2+Vit.C).

Stepping Test

The time taken to initiate movement with the four limbs was used to measure bradykinesia. Haloperidol induced parkinsonism (-D2) (30.80 ± 12.28 sec) caused a significant ($p < 0.05$) increase in the time

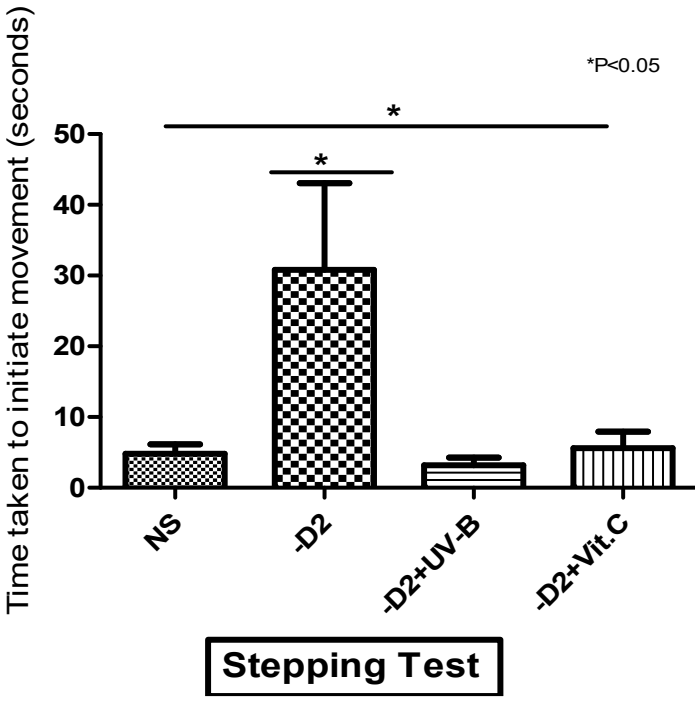

Figure 2. Stepping Test for Bradykinesia in Haloperidol Induced Parkinsonism (HIP), UV-B Treated HIP and Vit.C Treated HIP. * Significantly different compared with the control at p<0.05. UV-B group (-D2+UV-B) improved time taken to initiate movement, significantly when compared to –D2 (p<0.05).

taken to initiated movement compared with the control (NS) (4.80 ± 1.36 sec). Both UV-B rays (–D2+UV-B) (3.20 ± 1.07 sec) and vit. C treated

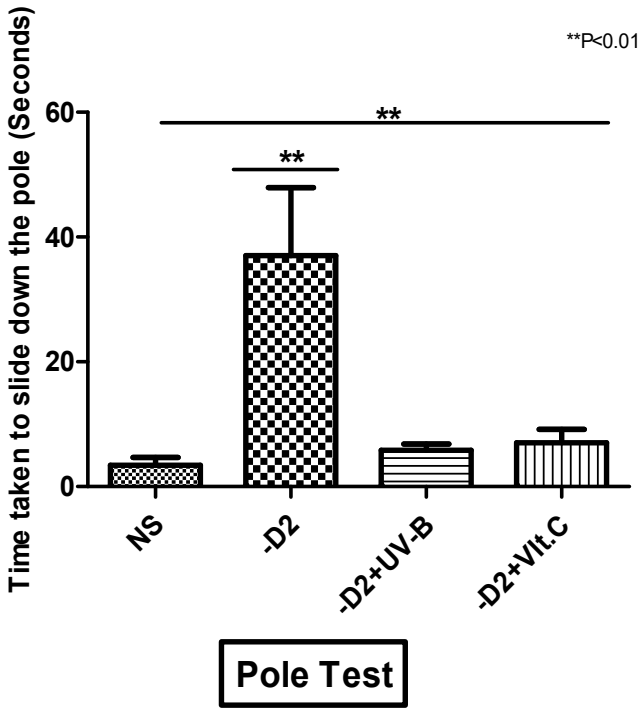

Figure 3: Pole test for assessment of rigidity in Haloperidol induced Parkinsonism (HIP), UV-B (-D2+UV-B) and vit. C intervention (-D2+Vit.C). ** Significantly different compared with the control at p < 0.01.

parkinsonism (-D2+Vit.C) (5.60 ± 2.36 sec) showed no significant difference in the time taken to initiate movement when compared to the control (NS).
However, when compared to untreated parkinsonian group (–D2), UV-B treated parkinsonian group (-D2+UV-B) showed a significant improvement (p < 0.05) and there was no significant different in Vit.C treated parkinsonian (–D3+Vit.C) when compared to -D2 group. Nevertheless, there was no significant difference between both UV-B and vit. C treated parkinsonism.

Pole Test
The time taken to slide down the smooth wooden pole was used to measure the degree of stiffness of the limb (rigidity). Haloperidol induced parkinsonism -D2 (37.00 ± 10.95 sec) caused significant (p < 0.01) increase in the time taken for the rats to slide down the pole compared to the control (NS) (3.40 ± 1.29 sec). There was no significant difference between both UV-B treated parkinsonism (-D2+UV-B) (5.80 ± 1.02 sec) and vitamin C treated parkinsonism (-D2+Vit.C) (7.00 ± 2.17 sec) compared with the control (NS).
When compared with the untreated parkinsonian group (-D2), both UV-B treated parkinsonism (-D2+UV-B) and vitamin C treated parkinsonism (-D2+Vit. C), showed significant improvement in the time taken to slide down the pole (p < 0.01).

Neurohistology of the Striatum (H&E Stain)
The control group revealed normal small and medium spiny neurons of striatum (Figure 4a). Small and medium spiny neurons of striatum of the untreated group showed several degenerating neurons which are characterized with cavitations, shrunken cell bodies, pyknotic bodies and extruded nuclei as seen in (Figure 4b). Both UV-B and vitamin C intervention did not show any striking difference (Figure 4c and d).

Neurohistology of the Motor Cortex (H&E Stain)
The motor cortex of the control group showed normal pyramidal and stellate (Figure 5a). –D2 demonstrated numerous cells with many of them having degenerated or unclear nuclei or even no nuclear. It also shows numerous shrunken eosinophilic neuronal cell bodies with several neurophilic vacuolisations (Figure 5b). UV-B rays intervention group (–D2+UV-B) revealed numerous shrunken cell bodies (Figure 5b). Cells having clear nuclei, neutrophilic vacuolizations were also seen in the group (Figure 5c). Vitamin C intervention group (–D2+Vit.C) showed numerous vacuolisations and cell body shrinkage (Figure 5d).

Neurohistology of Substantia Nigra (Cresyl Violet Stain)
The control group expressed normal histology of the substantia nigra, with clearly defined aggregated and brindled neuronal Nissl bodies (Figure 6a). Untreated

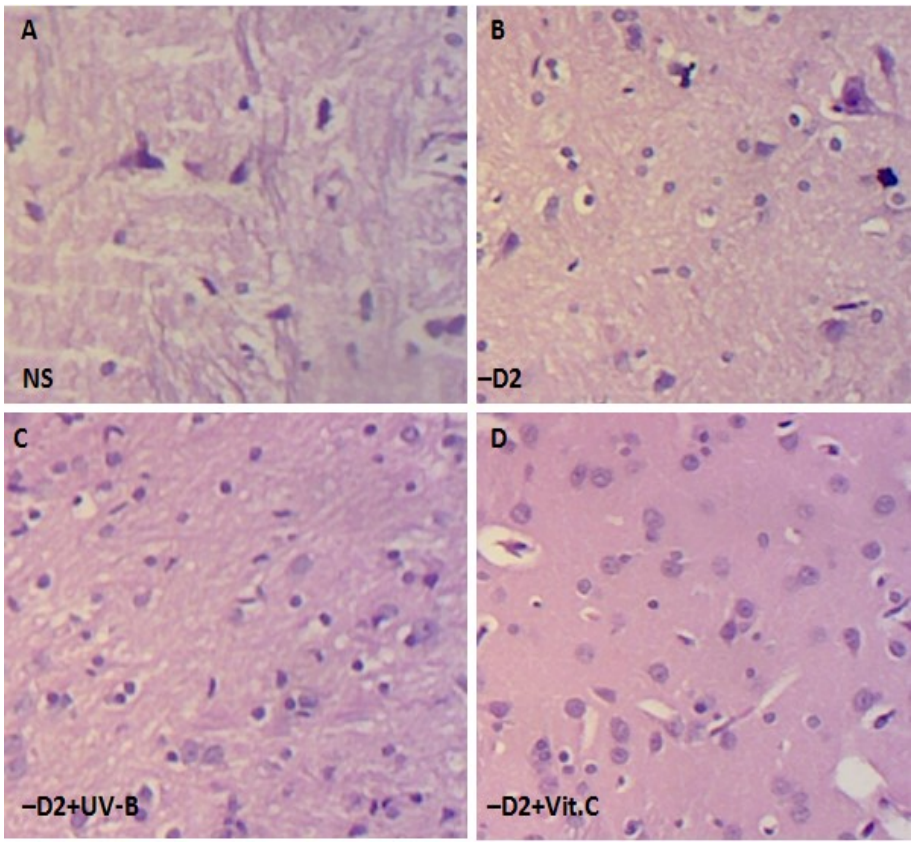

Figure 4a-d: Photomicrograph Showing Over-View of the Corpus Striatum. There is no striking difference across the groups. H&E, ×100).

parkinsonian group (–D2) showed densely populated, poorly stained and equivocal neuronal Nissl bodies (Figure 6b). There was no striking difference between the control group and UV-B rays intervention group (–D2+UV-B) (Figure 6b and 6c). Vitamin C intervention group (–D2+Vit.C) showed remarkable improvement in the organization of the neuronal Nissl bodies (Figure 6d).

Neurohistology of the Motor Cortex (Cresyl Violet Stain)

The control group showed unremarkable histological features (Figure 7a). Untreated parkinsonain group (–D20 expressed severe neurodegenerative signs including vacuolations and cavitation with intensely stained eosinophilic cytoplasm and tinctorial alterations (Figure 7b). UV-B rays intervention group (–D2+UV-B) showed vacuolization but with less severe cavitations (Figure 7c). Vitamin intervention group (–D2 +Vit.C) showed remarkable improv ement when compared to –D2 and –D2+UV-B. However it revealed intensely stained eosinophilic cytoplasm (Figure 7d).

Neurohistology of the Striatum (Cresyl Violet Stain)

The caudate nucleus and putamen (striatum) of the control group revealed unremarkable histology of striatum with numerous heteromorphic Nissl granules (Figure 8a). Untreated parkinsonian group (–D2) showed numerous cavitations and fewer Nissl substance (Figure 8b). There was no striking diff-

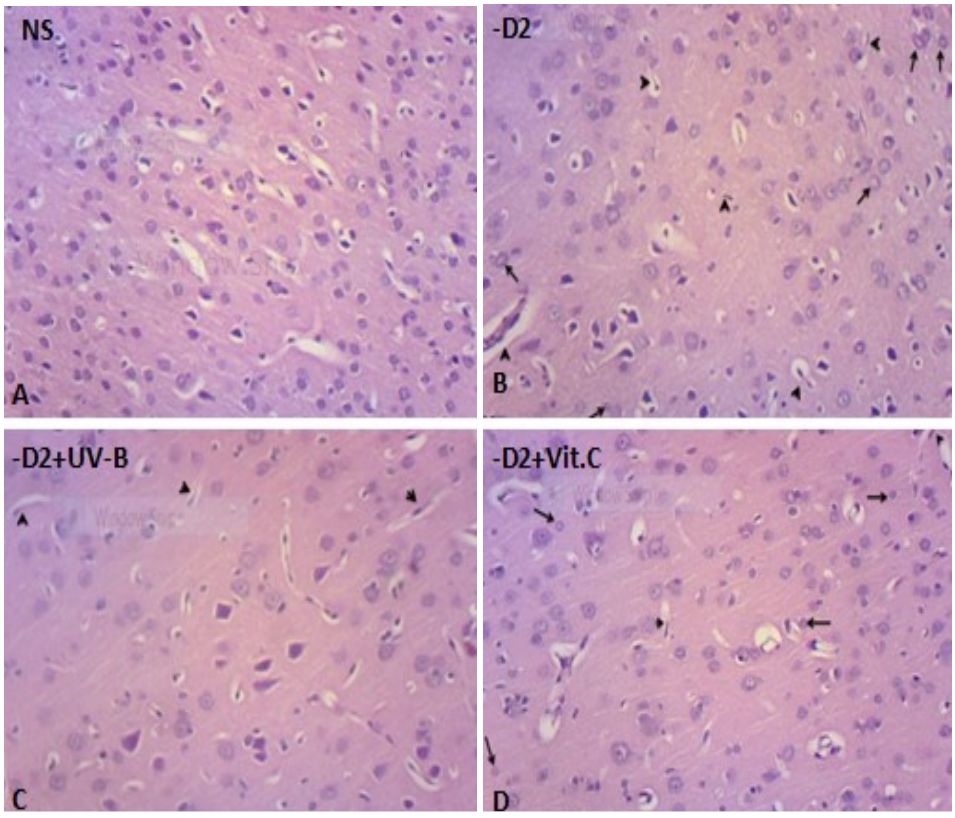

Figure 5a-d: Photomicrograph Showing Overview of the Layer 5 and 6 of Motor Cortex. NS shows normal pyramidal and stellate neural population.–D2 shows numerous cells with many of them having degenerated, unclear or no nuclei (arrow). There are numerous shrunken eosinophilic neuronal cell bodies (arrow head) with several neurophilic vacuolisation. –D2+UV-B group shows numerous shrunken cell bodies (arrow head), but the cells have clear nuclei. neutrophilic vacuolization. –D2+Vit.C shows numerous vacuolisation and cell body shrinkage. H&E,. ×100

erence between control group and UV-B rays intervention group (–D2+UV-B) (Figure 8b and 8c). Vitamin C intervention group (–D2+Vit.C) showed less cavitations with monomorphic Nissl granules as seen in (Figure 8d).

Nissl Bodies Counting

Nissl bodies loss compared to control (178.0 ± 19.08) was used to measure the degree of neuronal degeneration and regeneration was accessed by comparing –D2+UV-B (83.33 ± 29.92) and –D2+Vit.C (107.3 ± 15.33) with –D2 (52.33 ± 3.33). Haloperidol Induced parkinsonism (-D2) caused a significant loss of Nissl substance (degeneration) compared with the untreated control group (NS) ($p < 0.01$). UV-B treated parkinsonism (-D2+UV-B) showed less severe loss of cell bodies (degeneration) ($p < 0.05$). There was no significant lost of Nissl substance (degeneration) in vit. C treated parkinsonism group compared to the control (NS). Unfortunately, there was no significant increase in Nissl substances (regeneration) in both -D2+UV-B and or –D2+Vit C group compared to –D2.

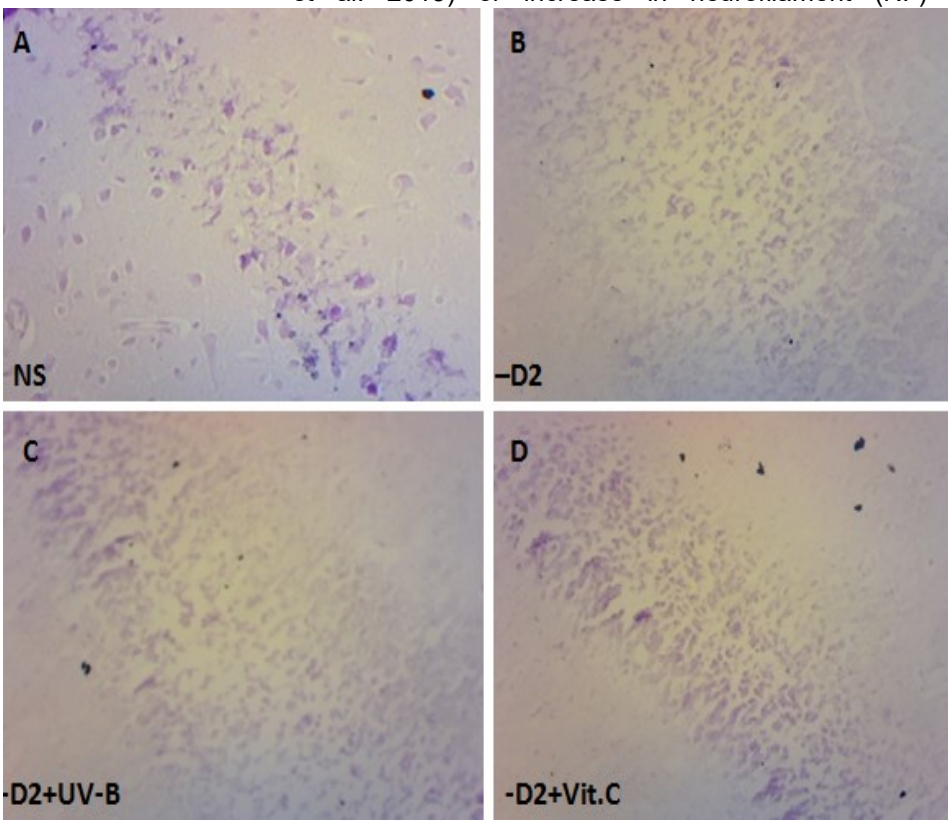

Figure 6a-d: Histological Demonstration of Nissl substance in the Substantia Nigra of Wistar Rats. NS represent normal histology of the substantia nigra showing clearly defined aggregated and brindled neuronal Nissl bodies. –D2 shows densely populated, poorly stained and equivocal neuronal Nissl bodies. –D2+UV-B group shows morphological changes similar to –D2. –D2+Vit.C shows improvement in the organization of the neuronal Nissl bodies. Cresyl violet, ×100

DISCUSSION

UV-B Rays and Vitamin C Treatment Improve Motor Function in Haloperidol Induced Parkinsonian Rats Model

Behavioural studies showed that prolonged interperitonial (i.p) administration of 10 mg/kg of haloperidol caused a significant decrease in motor activity of Wistar rats. This has been attributed to loss of synaptic connections, inhibition of dopamine receptors, loss of dopaminergic signaling and increase in expression of autophagosomes in the neurons (Booth et al. 2014; Kozina et al. 2014; Pifl et al. 2014).

UV-B rays caused a significant improvement of motor functions compared with the untreated parkinsonian group. This was observed as increase in climbing attempts (cylinder test; Figure 1), decr-ease in time taken to initiate steps (stepping test; Figure 2) and decrease in time taken to slide down the pole (Pole test; Figure 3). This may be as a result of vitamin D3 receptor activation (VD3RA) from UV-B rays. The improvement of motor coordination by VD3RA after acute drug induced parki-nsonism is caused by decre-asing M1 neural output and increasing CPu burst freque-ncies or change in extracellular calcium hyperpolarization current in the M1 and CPu (Ishola et al. 2015) or increase in neurofilament (NF) deposition (Holick, 2006) or enhancing calcium channel signalling in the brain (Yers, 2013).

Vitamin C treated parkinsonism (-D2+Vit.C) also improved the motor coordination of DIP similar to UV-B rays as observed in (cylinder, stepping and pole test; Figure 1, 2 and 3 respectively). Based on the studies that have shown that haloperidol induces oxidative stress in the rat brain (Polydoro et al. 2004) and vitamin C is a strong antioxidant (Pada- yatty, 2003), the motor deficit amelioration by vitamin C as obse-rved in the behavioural studies was deduced to be caused by reduction of oxidative stress level or modulation of dopamine trans-mission (Angelow et al. 2003).

Symptomology of Haloperidol Induced Parkinsonism (HIP)

HIP was not attributed to resting tremor as it was not observed in cylinder test during the climbing attempts. This reaffirms the studies of Booth et al. (2014) which show that drug-induced parkinsonism is less likely to be associated with tremors. However, the general

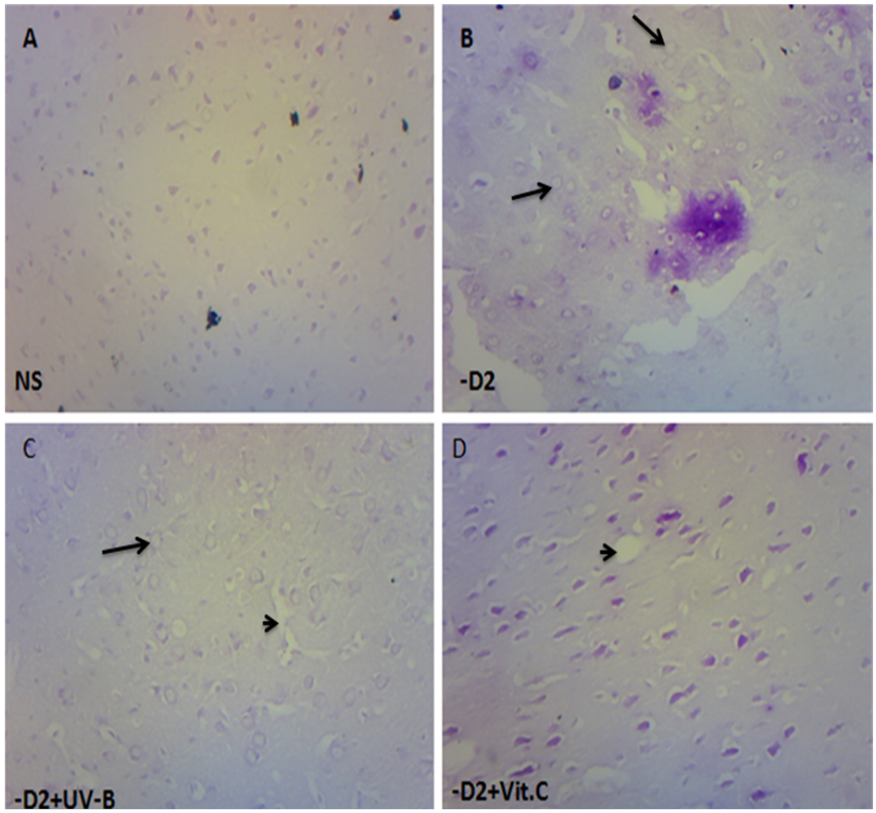

Figure 7a-d: Histological Representation of Nissl Substance in Layer 5 and 6 of Motor Cortex of Wistar Rats. NS shows normal histological features. –D2 shows severe neurodegenerative signs including vacuolations (arrow) and cavitation (arrow head) with intensely stained eosinophilic cytoplasm and tinctorial alterations. –D2+UV-B shows vacuolization but with less severe cavitations. –D2+Vit.C shows remarkable improvement compared to –D2 and –D2+UV-B but with intensely stained eosinophilic cytoplasm.. Cresyl violet, ×100

and torso leading to the increase in resistance to slide down the pole. These pheno-types are regarded as rigidity in Parkinsonism (Broussolle et al. 2007). This is also similar to the findings of Lorenc-Kochi et al. (1996) that haloperidol induced rigidity similar to that seen in parkinsonism by increased muscle tone in rats. Meltzer et al. (2003) found that drug induced parkinsonism is static i.e neither progress nor reverse upon withdrawal. Considering this finding, the chances of the positive effects being as result of haloperidol withdrawal are slim.

Neurodegenerative Changes Caus-ed by Prolonged Inh-ibition of Dopamine-2 Rec-eptor were not Successfully Reversed by UV-B Rays and Vit. C Treatment

The morphological studies revealed that HIP caused several types of neurodegen-erative hallmarks which

motor coordination tested in the cylinder test reduced significantly. In stepping test, HIP was found to be associated with akinesia (motor block) as seen in 3/5 of rats in -D2 group and difficulty to initiate movement. Berardelli et al. (2001) found that this motor abnormality is typically associated with basal ganglia disorder. Booth et al. (2014) found the same phenotype to be resulted from disruption of normal motor cortex activity as result of reduction of dopamine functions. In the pole test, it was found that HIP manifest tightness and inflexibility of the limb

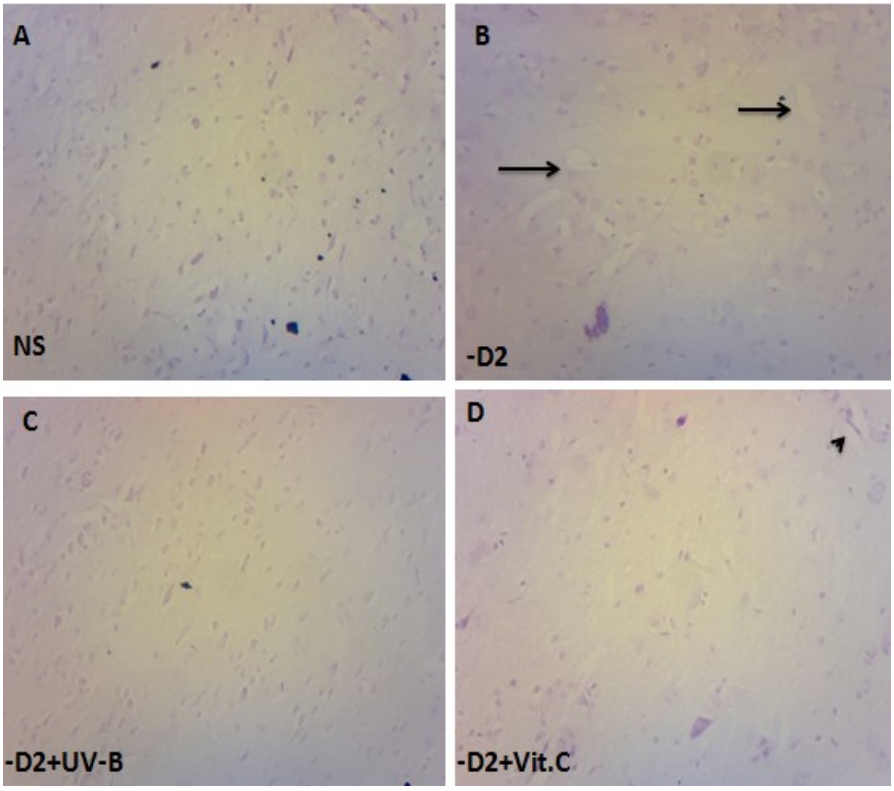

Figure 8a-d: Histological Repre-sentation of Nissl Substance in the Caudate Nucleus and Puta-men of Wistar Rats. NS represent normal histology of striatum with numerous heteromorphic Nissl granules. –D2 shows numerous cavitations and fewer Nissl substa-nce. –D2+UV-B appears similar to the control (NS). –D2+Vit.C shows less cavitations with monomorphic Nissl granules. Cresyl violet, ×100.

inclu-ded cavitations, vacuolization, neurons shrinkage and pyk-nosis. The severity of the degeneration signs varies in motor controlling region of the brain. The effect of both UV-B and Vit. C treatment also differs in those regions.

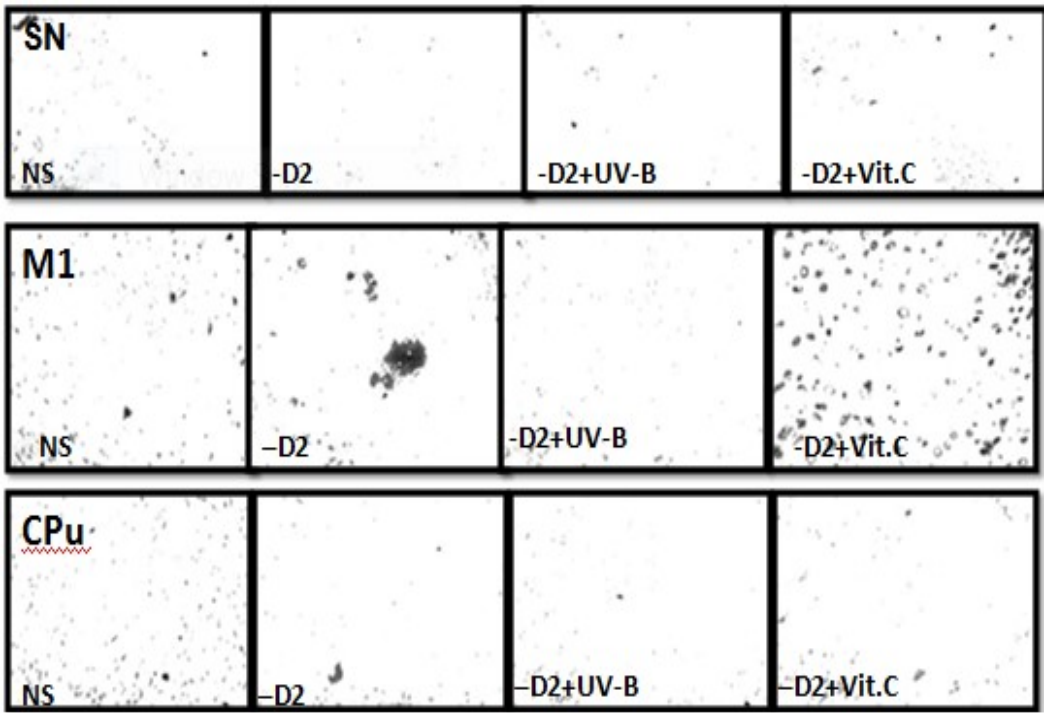

Figure 9a-c: ImageJ representation of substantial nigra, motor cortex and striatum of NS, -D2, -D2+UV-B and -D2+Vit.C groups after thresholding revealing only the normal granules.Cresyl violet, ×100

In substantia nigra, the equivocal, poorly stained and disorganized cell bodies observed in the SN of -D2 and -D2+UV-B group was one of the factors responsible for the phenotypes of the parkinsonism exhibited in the behavioural studies as the compacted and organized neurons of SN were found to be responsible for the motor control (Pioli et al. 2008)

Despite the improved motor activity of UV-B treated parkinsonism, it does not show any obvious improvement in the organization of the Nissl substances when compared with -D2 group as observed in (Figure 6b and 6c). However, vitamin C treated parkinsonism (Figure 6d) shows appreciable improvement in the general organization of the compacted Nissl substances when compared with the control (Figure 6a). This improvement is responsible for the improvement ascertained in the behavioral studies. Lewy bodies, the pathological hallmark of Parkinson's disease which are eosinophilic and can be observed under light microscope (Castellani et al. 2007) were not discovered in HIP. This indicates that HIP is less likely to be associated with Lewy bodies.

In striatum (caudate nucleus and putamen CPu), H&E stain does not reveal any striking differences across the groups.

In motor cortex, HIP was found to cause the appearance of several pyramidal and stellate cells with degenerated nuclei. M1 being responsible for initiation of voluntary movement (Singh 2009) the degeneration of the nuclei of the cells is responsible for akinesia and bradykinesia observed in stepping test.

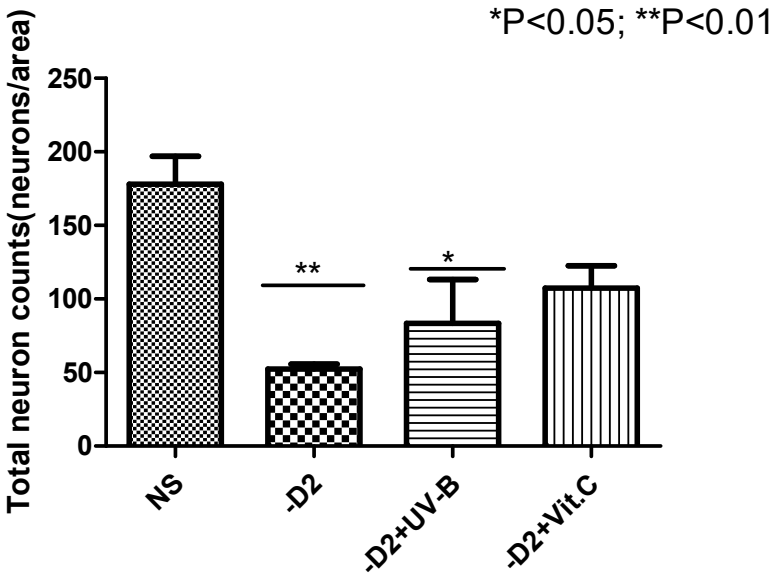

Figure 10. Nissl body count result of substantia nigra, striatum (CPu) and motor cortex of the experiment less cavitations with monomorphic Nissl granules. **, * Significant number of Nissl bodies were lost in –D2 and –D2+UV-B compared to the control at $p < 0.05$ and 0.01. Cresyl violet, ×100.

Nissl Bodies Count in Nigrostriatal and Corticostriatal Pathway (SNc+ CPu+M1)

Dissociation of ribosome from the RER occurs in the early stage of neurons degeneration. As a result, degenerating neurons actually stains very poorly (Robert 2011). For this reason, neurons in the early stage of degeneration were overlaid by the threshold done with imageJ. The Nissl body count result clearly indicates that prolonged administration of halop-eridol results into significant loss of

nissl bodies in substantia nigra, striatum and motor cortex (Jai et al. 2000) which resulted into the parkinsonism (Jung et al. 2014; Pifl et al. 2014) observed in the behavioural studies. Not having significant incr-ease in the number of Nissl bodies indicates that both UV-B and or Vit. C treatment do not restore the Nissl body loss inflicted by prolonged administration of haloperidol.

Conclusion

UV-B and Vit. C treatment show ameliorative potentials in reducing motor deficit exper-ience in Parkinsonism but not regenerating already lost neu-rons.

Conflict of Interest

None declared.

Author Contributions

SMU and IAO initiated and designed the study. LFO and MA supervised the study. SK and OOE participated in the implementation and design of apparatus for behavioural studies.

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