



Jobelyn® improves motor dysfunctions induced by haloperidol in mice via neuroprotective mechanisms relating to modulation of cAMP response-element binding protein and mitogen-activated protein kinase

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Abstract

The clinical efficacy of haloperidol in the treatment of psychosis has been limited by its tendency to cause parkinsonian-like motor disturbances such as bradykinesia, muscle rigidity and postural instability. Oxidative stress-evoked neuroinflammation has been implicated as the key neuropathological mechanism by which haloperidol induces loss of dopaminergic neurons and motor dysfunctions. This study was therefore designed to evaluate the effect of Jobelyn® (JB), an antioxidant supplement, on haloperidol-induced motor dysfunctions and underlying molecular mechanisms in male Swiss mice. The animals were distributed into 5 groups (n=8), and treated orally with distilled water (control), haloperidol (1 mg/kg) alone or in combination with each dose of JB (10, 20 and 40 mg/kg), daily for 14 days. Thereafter, changes in motor functions were evaluated on day 14. Brain biomarkers of oxidative stress, proinflammatory cytokines (tumor necrosis factor-alpha and interleukin-6), cAMP response-element binding protein (CREB), mitogen-activated protein kinase (MAPK) and histomorphological changes were also investigated. Haloperidol induces postural instability, catalepsy and impaired locomotor activity, which were ameliorated by JB. Jobelyn® attenuated haloperidol-induced elevated brain levels of MDA, nitrite, proinflammatory cytokines and also boosted neuronal antioxidant profiles (GSH and catalase) of mice. It also restored the deregulated brain activities of CREB and MAPK, and reduced the histomorphological distortions as well as loss of viable neuronal cells in the striatum and prefrontal cortex of haloperidol-treated mice. These findings suggest possible benefits of JB as adjunctive remedy in mitigating parkinsonian-like adverse effects of haloperidol through modulation of CREB/MAPK activities and oxidative/inflammatory pathways.

Keywords Haloperidol · Parkinsonian-like motor symptoms · Jobelyn® · CREB and MAPK pathways

Abbreviations

JB Jobelyn^(R)
PD Parkinson's disease
TBA Thiobarbituric acid

DTNB [5', 5'-Dithiobis- (2-nitrobenzoate)
TCA Trichloroacetic acid
HAL Haloperidol
GSH Glutathione
MDA Malondialdehyde
CREB cAMP response-element binding protein
MAPK Mitogen-activated protein kinase

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Introduction

Parkinson's disease (PD) is a neurological disorder due to progressive degeneration of nigrostriatal dopaminergic neurons and development of fibrillar cytoplasmic inclusions

of α -synuclein (McCormack et al. 2002; Qian et al. 2010; Schapira and Jenner 2011). The death of nigrostriatal dopaminergic neuron leads to loss of dopamine, and thus the manifestations of motor symptoms such as bradykinesia, muscular rigidity and postural imbalance (Qian et al. 2010; McCormack et al. 2002). Epidemiological data have established a linear relationship between exposures to environmental toxicants such pesticides (rotenone) and herbicides (paraquat), and the genesis of PD (McCormack et al. 2002). For example, high prevalence of PD has been reported in farmers who were exposed to pesticides and herbicides (McCormack et al. 2002). Incidentally, administration of rotenone and paraquat is being used to replicate the neuropathological features of PD in experimental animals (Dauer and Przedborski 2003; Bezard and Przedborski 2011; Alabi et al. 2019; Alam and Schmidt 2002). However, prolonged antipsychotic medications such as haloperidol are also known to induce extrapyramidal adverse effects akin to symptomatology of PD (Andreassen and Jorgensen 2000; Saeed et al. 2017; Zhuravliova et al. 2007; Sykes et al. 2017).

Indeed, the clinical efficacy of haloperidol for the treatment of psychosis has been severely compromised by its tendency to cause motor disturbances such as bradykinesia, muscle rigidity and postural instability, which are the most common debilitating symptoms of PD (Zhuravliova et al. 2007; Saeed et al. 2017; Sykes et al. 2017; Waku et al., 202). Haloperidol is also known to produce abnormal movements such as oral dyskinesia in animals, which has been linked to morphological alterations in distinct brain regions especially the striatum (Kelley et al. 1997; Halliday et al. 1999; Andreassen and Jorgensen 2000). However, catalepsy is one of the most prominent neurobehavioral deficits of haloperidol widely measured in laboratory animals due to its similarity to the symptoms of PD (Zhuravliova et al. 2007; Sykes et al. 2017; Waku et al. 2021). Catalepsy has been described as a state of bradykinesia and muscle rigidity, in which the animal cannot correct externally imposed abnormal postures (Sykes et al. 2017; Waku et al. 2021).

Oxidative stress, resulting from alterations of the mitochondrial electron transport chain, has been proposed as the main neuropathological mechanism through which haloperidol induces loss of dopaminergic neurons and extrapyramidal adverse effects (Saeed et al. 2017; Cadet and Kahler, 1994; Casey 2000; Post et al. 2002). Indeed, several studies have shown that haloperidol-induced neuronal damage is due to activation of reactive oxygen species (ROS) formation and reductions in the intracellular concentrations of glutathione (Shivakumar and Ravindranath 1993; Post et al. 1998, 2002; Yokoyama et al. 1998). The clinical symptoms of PD appear only when dopaminergic neuronal cell death exceeds a critical threshold of over 70% of striatal nerve

terminals and increased oxidative stress has been implicated as the key mediator of haloperidol-induced neuronal cell death (Saeed et al. 2017). Oxidative stress-induced neuronal injury is known to be further aggravated by the release of inflammatory mediators, thus, linking neuroinflammation and oxidative stress as co-conspirators in the pathology of PD (Sutachan et al. 2012; Taylor et al. 2013). The findings of reduced antioxidant molecules, high concentration of oxidized lipids and protein in the brains of individuals with PD further confirmed the key role of oxidative stress in the pathology of this illness. Thus, the co-administration of agents with potent antioxidant property with haloperidol, might prevent the debilitating motor symptoms in psychotic patients on prolonged haloperidol medication. This supposition is based on the understanding that the inhibition of oxidative stress will prevent the multiple offensive mediators involved in haloperidol-induced neuronal cell death, which constitute the well-known concept of neuroprotection (Qian et al. 2010; Post et al. 1998; Schapira and Olanow 2004).

Jobelyn® (JB) is an African-based dietary supplement derived from the polyphenol-rich leaf sheath of *Sorghum bicolor* (Gramineae) that has been reported to exhibit wide range of neurobiological activities. For example, anti-amnesic, antioxidant and neuroprotective activities of JB have been established in various animal models (Umukoro et al. 2013; Oyinbo et al. 2015). It was also shown to antagonize psychotic-like symptoms induced by amphetamine and apomorphine without causing cataleptic adverse effect in mice (Omogbiya et al. 2013). In addition, we further reported that JB ameliorated neurological deficits induced by ischemic stroke in rats through neuroprotective mechanisms relating to antioxidant effect and downregulation of nuclear factor Kappa-B expression (Umukoro et al. 2018a, b). Recently, we also showed that JB extended the life span and improves motor dysfunction via augmentation of antioxidant status in *Drosophila melanogaster* exposed to lipopolysaccharide (John et al. 2022). Besides, HPLC investigations have shown that JB has biologically active constituents such as apigenidin, proapigenidin, apigenin, luteolin and naringenin (Geera et al. 2012; Benson et al., 2013; Makanjuola et al. 2016), which have been reported to exhibit neuroprotection and anti-neuroinflammation through various molecular mechanisms (Lou et al. 2014; Li et al. 2016; Zhang et al. 2017; Umukoro et al. 2018a, b; Olugbemide et al. 2021). The findings that JB and its phyto-constituents possess potent antioxidant and neuroprotective activities suggest that it might protect against haloperidol-induced neuronal cell death and motor dysfunctions, and thus reducing its debilitating motor symptoms in psychotic patients. This study was therefore designed to evaluate the ameliorative and neuroprotective effects of JB on haloperidol-induced motor dysfunctions, and underlying molecular mechanisms in male Swiss mice.

Materials and methods

Laboratory animals

Male Swiss mice (22–25 g) used in the study were purchased from the Central Animal House, University of Ibadan, Ibadan, Nigeria. They were acclimated in the Department of Pharmacology and Therapeutics Animal Holding Facility for one week before the commencement of the study. They were housed in polycarbonates fabricated animal cages with free access to standard rodent pellet diet (Vital feeds®, Jos, Nigeria) and water *ad libitum*. The ethical principles established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8523, revised 2011) were followed.

Drugs and chemicals

Jobelyn® (Health Forever Products Ltd, Lagos, Nigeria) and haloperidol (Sigma-Aldrich, St. Louis, USA) were used in the study. Reagents used include: trichloroacetic acid (TCA) (Burgoyne Burbidges & Co., Mumbai, India), thiobarbituric acid (TBA) (Sigma-Aldrich, St. Louis, USA), Ellman Reagent [5', 5'-Dithiobis- (2-nitrobenzoate) DTNB] (Sigma-Aldrich, St. Louis, MO, USA) and hydrogen peroxide (H₂O₂) (BDH Chemicals Ltd., Poole, England). ELISA kits include tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) from Bio Legend (USA), CREB ELISA Kit (Cat. No. EM0951) and MAPK ELISA Kit (Cat. No. EM1199) from Wuhan Fine Biotech Co., Ltd, China.

Drug preparation

Haloperidol was prepared according to the procedure earlier described (Ben-Azu et al. 2018a), whereas the method described by Omorogbe et al. 2018 was followed in preparation of JB. Briefly, haloperidol and JB were dissolved in distilled water on the experimental day. The dose of 1 mg/kg of haloperidol used in this study was chosen according to our previous studies wherein we observed significant cataleptic behavior resulting from degeneration of cortical pyramidal neurons (Ben-Azu et al. 2018b, c, d, 2019). The doses of 10, 20 and 40 mg/kg used for this study were selected based on the findings of Omorogbe et al. 2018.

Table 1 Treatments and experimental design

Group	Treatment & Dose
1.	Distilled water (10 mL/kg)
2.	Distilled water (10 mL/kg) + Haloperidol (1 mg/kg)
3.	Jobelyn® (10 mg/kg) + Haloperidol (1 mg/kg)
4.	Jobelyn® (20 mg/kg) + Haloperidol (1 mg/kg)
5.	Jobelyn® (40 mg/kg) + Haloperidol (1 mg/kg)

Treatments and experimental design

Male Swiss mice were randomly distributed into 5 groups (n = 8). Mice in group 1 were given distilled water (control), group 2 also received distilled water (haloperidol-control) while groups 3–5 had JB (10, 20 and 40 mg/kg, p.o.), daily for 14 days. Thirty minutes after each daily oral treatment, mice in groups 2–6 were given haloperidol (1 mg/kg, p.o). The changes in motor functions were assessed on day 14. The brain biomarkers of oxidative stress, proinflammatory cytokines (tumor necrosis factor- α and interleukin-6), CREB, MAPK and histomorphological changes were also determined (Table 1).

Behavioral procedures

Open field test

The animals were placed individually in the center of the open field chamber (72 cm×72 cm×36 cm). Then, the number of lines crossed were recorded by a trained blinded observer for a period of 5 min as previously described (Ben-Azu., 2018a).

Catalepsy test

The effect of JB on haloperidol-induced catalepsy was investigated according to the modified version as earlier described (Costall et al., 1974). The test was done by gently placing the fore limbs of each animal on a horizontal plane wood surface (H = 6 cm; W = 4 cm; L = 16 cm) and the duration of akinesia (period of time the animal remained in one position, before initiating any active movement) in seconds was recorded.

Walk beam test

This test is commonly used for evaluating motor coordination and postural stability in rodents and the procedure earlier described by Omorogbe et al. (2018) was adopted in this study. The walk beam consists of one-metre (100 cm) long beam with a flat surface of width 15 by 7.5 mm elevated to a height of 40 cm between two wooden supports. Beginning at the 15-mm mark, the mice were placed individually on the unsheltered end and allowed to traverse across the beam to the other end. The number of paw slips and distance travelled was recorded for a period of 5 min (Omorogbe et al. 2018).

Pole test

The pole test is a simple model for assessing motor function in rodents and the test is premised on the ability of the

mouse to orient itself downwards and descend to the base of the pole when positioned head-up on top of a 60-cm pole of 1-cm diameter. Mice were assessed for the times taken to turn their entire body downwards towards the base (turn time) and descend without sliding (descent time) as earlier described (Saeed et al. 2017).

Preparation of brain tissues for biochemical assays

The animals ($n=5$) were euthanized using diethyl ether and each of the isolated brain was weighed and rinsed with 10% w/v sodium phosphate buffer (0.1 M; pH 7.4). Then, each brain tissue was homogenized and centrifuged, and supernatant collection was done using previous established procedures (Ben-Azu et al. 2018a).

Assay for oxidative stress parameters and nitrite content

The level of MDA was estimated in the brain tissue using the thiobarbituric reacting substance (TBARS) assay (Ohkawa et al. 1979). The brain concentrations of MDA were calculated as previously described (Ohkawa et al. 1979) and expressed as $\mu\text{mol MDA/g tissue}$. The nitrite content was determined using the protocol described by Green et al. (1981). The absorbance was read at 540 nm and the nitrite content was then estimated from the standard curve of sodium nitrite (0–100 M). The GSH content was estimated as earlier described (Moron et al. 1979) and expressed as $\mu\text{M GSH/g tissue}$. Catalase activity was estimated using the procedure of Goth (1991) and expressed as $\mu\text{moles of hydrogen peroxide decomposed per min per mg protein (Unit/mg protein)}$. The method of Misra and Fridovich (1972) was followed for the assay of SOD activity (Unit/mg protein). Protein concentration was determined using the procedure of Lowry et al. (1951).

Estimation of TNF- α , IL-6, MAPK and CREB levels

The levels of TNF- α , IL-6, MAPK and CREB in the brain's supernatants were measured based on the manufacturer's protocols. They were measured using specific mouse TNF- α (Bio Legend ELISA MAX™ Deluxe kit, USA), IL-6 (Bio-Legend ELISA MAX™ Deluxe kit, USA), CREB (Wuhan Fine Biotech Co., Ltd, China) and MAPK (Wuhan Fine Biotech Co., Ltd, China). The measurements were done at room temperature in accordance to manufacturers' instructions using Microplate reader (LT-4500, UK) at 450 nm. The TNF- α , IL-6, CREB, and MAPK concentrations from each brain tissue was extrapolated from their standard curves. The levels of TNF- α and IL-6 were expressed as pg/mg protein while CREB, and MAPK concentrations were presented as ng/mg protein .

Preparation of brain tissues for histology

Mice ($n=3$) in the respective treatment groups were anaesthetized with ether. Thereafter, the mice were perfused, and their brains were harvested, and fixed with 10% phosphate buffered formaldehyde. The brains were then subjected to the routine method for paraffin wax embedment to obtain paraffin wax embedded tissue blocks. Given that haloperidol was previously reported to cause degeneration of the nigrostriatal pathway and cortical pyramidal neurons of the prefrontal cortex (PFC) and hippocampus (Ben-Azu et al. 2019; Osacka et al. 2022), transverse sections (5–6 μm thick) of the striatum, PFC and hippocampal (cornu ammonis 3, CA1) regions were obtained with the aid of microtome (Leica, Germany) and the sections were fixed on glass slides. Viable neuronal cells were estimated using research microscope (Olympus CH (Japan) at x 960 magnification and graticule, and quantified as earlier described (Ben-Azu et al. 2019, 2022). Viable neurons were regarded as well rounded-shaped, intact cytoplasmic cells, without obvious distortions (Emokpae et al. 2020).

Statistical analysis

After normality and homogeneity data checks, data were expressed as $\text{mean} \pm \text{S.E.M}$ (standard error of mean) and analyzed using Graph Pad Prism software version 9.00. Statistical analysis of data was done using one-way ANOVA, followed by Bonferroni post-hoc test. P-values less than 0.05 ($p < 0.05$) were considered statistically significant.

Results

Jobelyn® ameliorates haloperidol-induced motor dysfunctions in mice

The effects of JB on haloperidol-induced motor impairments in mice are shown in Fig. 1A–D. One-way ANOVA showed that there were significant differences between treatment groups: number of lines crossed, distance traversed, and duration of catalepsy. Bonferroni post-hoc test revealed that haloperidol significantly reduced locomotor activity and distance traversed on the walk beam when compared with controls (Fig. 1A–B). It also produced a significant ($p < 0.001$) increase in the duration of catalepsy and impaired turning and descending behavior of mice in the vertical pole test when compared with controls (Fig. 1C–D). As shown in Fig. 1A–D, JB attenuated impairment of locomotor activity and motor incoordination induced by haloperidol in mice.

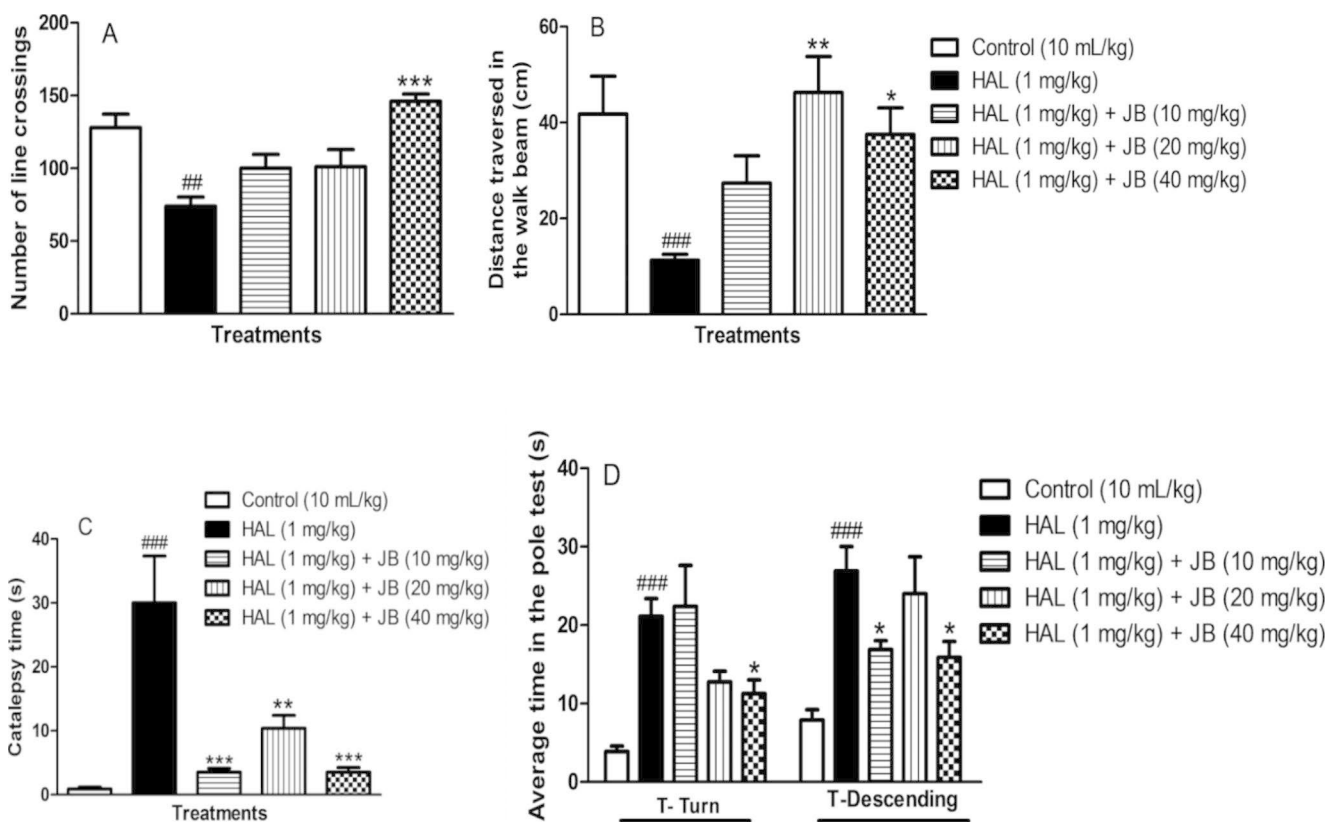
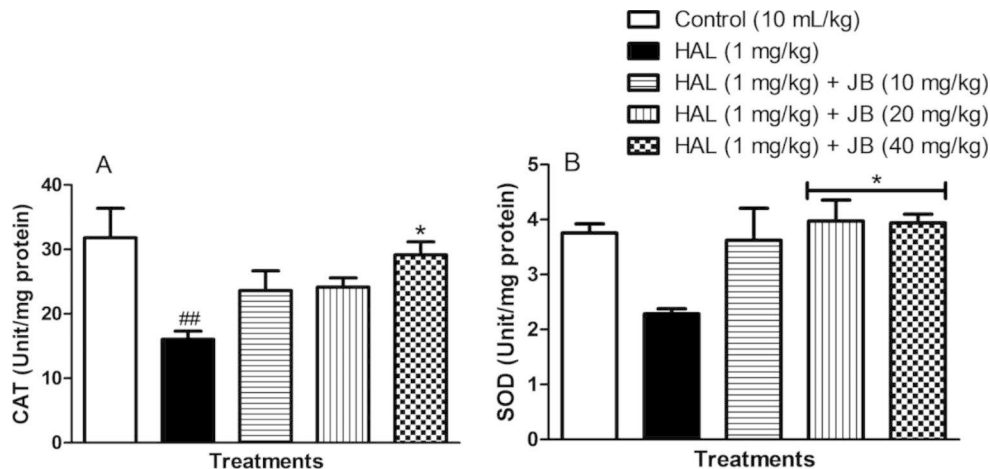


Fig. 1 Jobelyn® (JB) ameliorates haloperidol (HAL)-induced motor impairments in mice subjected to open-field (A), walk beam (B), wood block (C) and pole (D) tests. Bars represent the mean ± S.E.M of 8 ani-

mals per group. ##*p* < 0.01, ###*p* < 0.001 compared to control group, **p* < 0.05, ***p* < 0.01, ****p* < 0.001 compared to HAL group (one-way ANOVA followed by Bonferroni *post-hoc* test)

Fig. 2 JB elevates brain antioxidant enzyme activity: catalase (A) and superoxide dismutase (B) in mice exposed to HAL. Bars represent the mean ± S.E.M of 5 animals per group. ##*p* < 0.01 compared to control group; **p* < 0.05 compared to HAL group (one-way ANOVA followed by Bonferroni *post-hoc* test)



Jobelyn® increases brain antioxidant enzymes in mice exposed to haloperidol

One-way ANOVA and *post-hoc* tests indicate that administration of haloperidol (1 mg/kg, *p.o.*) caused a significant decrease in CAT activity. However, no significant change was observed in the levels of SOD activity in comparison with controls (Fig. 2A-B). Notably, JB (40 mg/kg, *p.o.*) significantly increased CAT (*p* < 0.01) activity relative to haloperidol (Fig. 2A).

Jobelyn® restores altered brain contents of GSH, MDA and nitrite in mice exposed to haloperidol

The effects of JB on brain concentrations of GSH, MDA and nitrite in mice exposed to haloperidol are shown in Fig. 3A-C. One-way ANOVA analysis showed that there were significant differences between treatment groups: GSH, MDA and nitrite (Fig. 3A-C). As presented in Fig. 3A-C, haloperidol significantly increased MDA and nitrite brain

contents accompanied by depletion of GSH in haloperidol-treated animals. However, JB (40 mg/kg, p.o.) significantly decreased MDA and nitrite, and elevated GSH brain concentrations of mice exposed to haloperidol (Fig. 3A-C).

Jobelyn® reduces brain contents of TNF- α and IL-6 in haloperidol-treated mice

Administration of haloperidol (1 mg/kg, p.o.) significantly increased TNF- α and IL-6 levels when compared with controls (Fig. 4A-B). Jobelyn® (20 and 40 mg/kg, p.o.) significantly ($p < 0.05$, $p < 0.01$) reduced the increased TNF- α concentration in mice brains treated with haloperidol (1 mg/kg, p.o.) in comparison with haloperidol group (Fig. 4A). As regards IL-6, JB (40 mg/kg, p.o.) significantly reduced brain concentration of IL-6 when compared with haloperidol group (Fig. 4B).

Jobelyn® modulates brain CREB and MAPK activities in mice treated with haloperidol

Haloperidol significantly decreased CREB levels when compared with control (Fig. 5A). But, JB (20 and 40 mg/

kg, p.o.) significantly up-regulated CREB activity when compared with haloperidol group (Fig. 5A). As shown in Fig. 5B, haloperidol significantly increased MAPK activity ($p < 0.001$) when compared with control, which was significantly ($p < 0.05$) down-regulated by JB (40 mg/kg, p.o.).

Jobelyn® improves histomorphological distortions and neuronal cell viability of haloperidol-treated mice

The photomicrographs of the effect of JB on haloperidol-induced histomorphological changes and density of viable cells of the striatum, PFC and hippocampus (CA1) of mice are shown in plate and Fig. 6. Nissyl staining revealed that haloperidol (1 mg/kg, p.o.) caused a severe morphological atrophy of neurons of the striatum and PFC (Fig. 6). Also, haloperidol induced a significant increase in the population of pyknotic neurons in the striatum and PFC when compared to control, which were prevented by JB (20 and 40 mg/kg, p.o.) (Fig. 7). However, no significant changes were observed in the CA1 region of the hippocampus following treatments with haloperidol relative to control (Figs. 6 and 7).

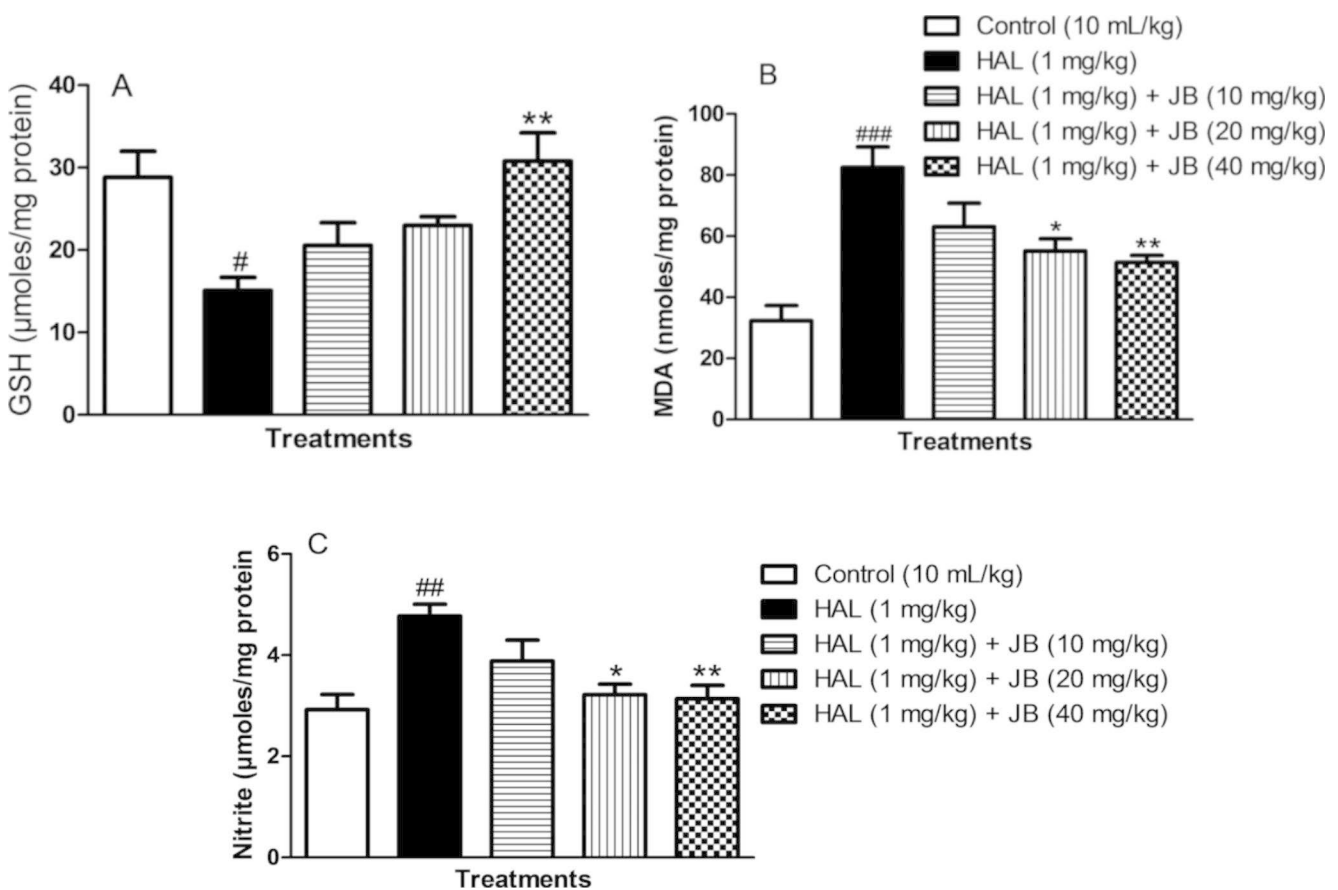


Fig. 3 JB attenuates haloperidol HAL-induced oxidative and nitroergic stress in mice brains: Glutathione, GSH (A), malondialdehyde, MDA, (B) and nitrite (C). Bars represent the mean \pm S.E.M of 5 animals / group. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to control group; * $p < 0.05$, ** $p < 0.01$ compared to HAL group (one-way ANOVA followed by Bonferroni *post-hoc* test)

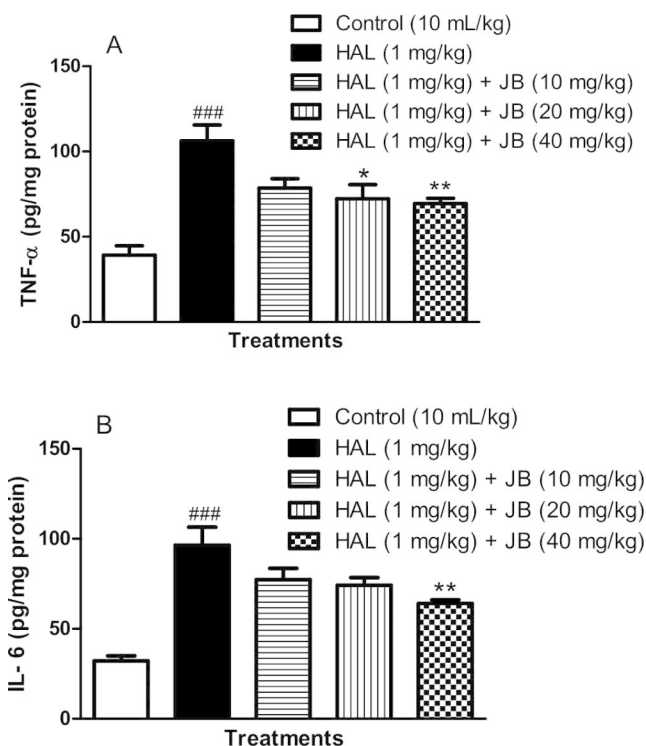


Fig. 4 JB reduces brain concentrations of tumor necrosis factor- α (TNF- α) (A) and interleukin-6 (IL-6) (B) in mice exposed to HAL. Each column represents the mean \pm S.E.M of 5 animals / group. ### $p < 0.001$ compared to control group; * $p < 0.05$, ** $p < 0.01$ compared to HAL group (one-way ANOVA followed by Bonferroni *post-hoc* test). HAL=Haloperidol, JB=Jobelyn®. *Tumor necrosis factor-alpha* = TNF- α , *Interleukin-6* = IL-6.

Discussion

Oxidative stress, resulting from alterations of the mitochondrial electron transport chain, has been proposed as the main neuropathological mechanism through which haloperidol induces loss of dopaminergic neurons and extrapyramidal adverse effects (Saeed et al. 2017; Cadet and Kahler 1994; Casey 2000; Post et al. 1998). Indeed, several studies have shown that haloperidol-induced neuronal damage is due to activation of reactive oxygen species (ROS) formation and depletion of intracellular concentrations of glutathione (Post et al., 1996; Post et al. 2002; Shivakumar and Ravindranath 1993; Yokoyama et al., 1993). Thus, it has been proposed that the reduction of oxidative stress overload through administration of free radical scavengers might be a rational approach in mitigating the adverse effects of prolonged haloperidol therapy (Post et al. 1998; Schapira and Olanow 2004; Qian et al. 2010), hence, the use of JB in this study. The data obtained from this study showed that haloperidol produced impairment of motor functions as evidenced by reduced locomotor activity, increased cataleptic period and postural instability in mice. However, treatment with JB ameliorated the parkinsonian-like motor symptoms induced by haloperidol in mice.

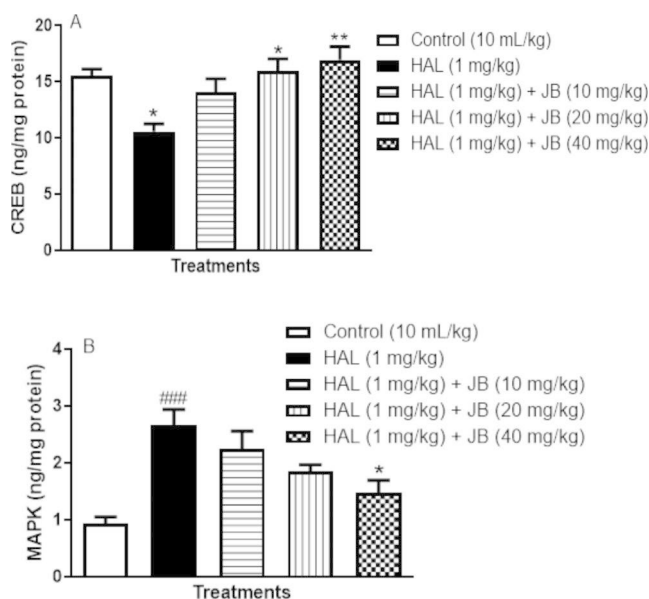


Fig. 5 JB up-regulates cAMP response-element binding protein (A) and reduces mitogen-activated protein kinase (B) activities in brains of mice exposed to HAL. Bars represent the mean \pm S.E.M of 5 animals / group. # $p < 0.05$, ### $p < 0.001$ compared to control group; * $p < 0.05$, ** $p < 0.01$ compared to HAL group (one-way ANOVA followed by Bonferroni *post-hoc* test). cAMP response-element binding protein = CREB, Mitogen-activated protein kinase = MAPK, HAL = Haloperidol, JB = Jobelyn®.

Earlier studies have shown that administration of haloperidol to laboratory animals induced parkinsonian-like disorders such as catalepsy and postural instability (Saeed et al. 2017; Sharma et al. 2017; Waku et al. 2021). Catalepsy is described as the inability of an animal to correct an externally imposed posture (Saeed et al. 2017; Waku et al. 2021). A normal animal is known to correct itself immediately after being placed in an abnormal position whereas a cataleptic animal remains in the imposed posture for a longer period of time (Waku et al. 2021). Thus, cataleptic state is a reflection of motor dysfunction leading to difficulty in initiating voluntary movement as exhibited by patients with PD. The finding that JB attenuated the cataleptic disorder induced by haloperidol suggests that it might mitigate the motor disturbances caused by prolonged use of this medication by psychotic patients.

The vertical pole test, walk beam model and open filed test were further used to evaluate the effects of JB on motor deficits induced by haloperidol in mice. The pole test, which was originally developed to evaluate bradykinesia in mice is based on the tendency of rodents to orient themselves downwards and descend the pole without sliding off (Saeed et al. 2017). The vertical pole test is often used to measure the sensorimotor function of rodents, which is another key neurological function, grossly impaired in patients with PD (Saeed et al. 2017). The result of this study is in agreement

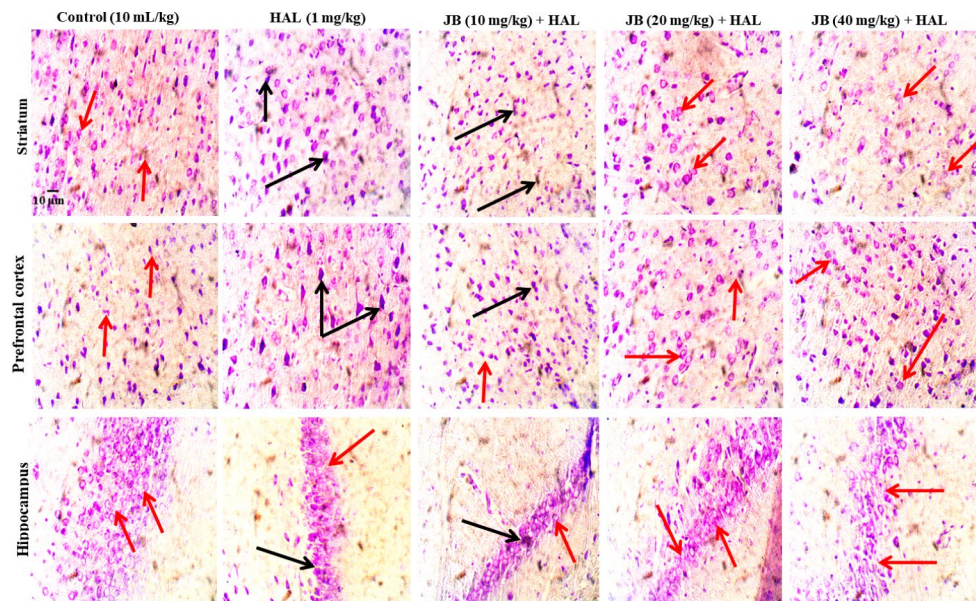


Fig. 6 Representative photomicrographs of the effect of JB on the *striatum*, *prefrontal cortex (PFC)* and *hippocampus (CA1)* of mice brains treated with HAL: In the striatum and PFC, control slides revealed no atrophic lesions but normal multiple neuronal cells patterns. Haloperidol (HAL) (1 mg/kg) slides show population of neurons with atrophy, dark chromatin nuclei and pyknosis. Jobelyn® (JB) (10 mg/kg)+haloperidol (1 mg/kg) slides show moderate atrophy and neuronal deaths. JB (20 and 40 mg/kg)+HAL (1 mg/kg) slides showed cells with no observable atrophy and lesions having increased population of viable cells. However, in the hippocampus, control slide showed no atrophic lesions but normal multiple neuronal cells without pyknosis. But, in JB (10 mg/kg)+HAL (1 mg/kg) slides there were mild atrophy with increased population of normal neuronal cells. In the JB (20 and 40 mg/kg)+HAL (1 mg/kg) slides, no observable atrophy was found. *Black arrow indicates normal neurons with no atrophy; red arrow indicates pyknotic neurons with atrophy.* Nissyl stain: x400. Calibration bar = 0.01 mm (10 µm) for all figures

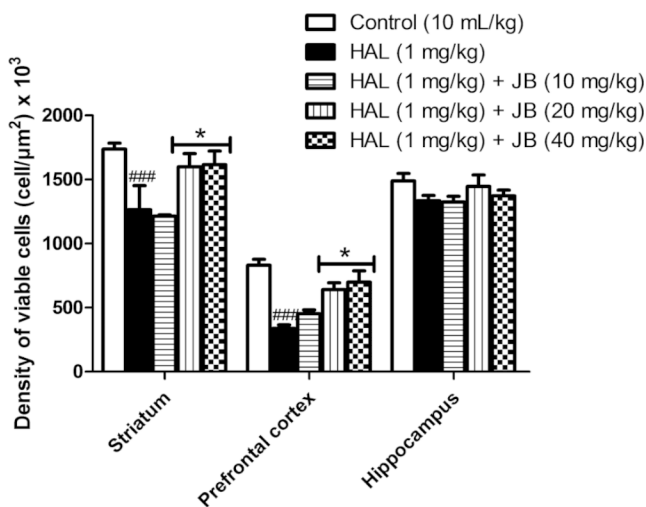


Fig. 7 Densitometric score of the effects of jobelyn® (JB) on the histomorphology of the striatum, prefrontal cortex (PFC) and hippocampus of mice treated with haloperidol (HAL). Bars represent the mean \pm S.E.M of 3 animals / group. ### $p < 0.001$ compared to control group; * $p < 0.05$ compared to HAL group (one-way ANOVA followed by Bonferroni *post-hoc* test)

with earlier investigations (Waku et al. 2021), which showed that mice treated with haloperidol exhibited difficulty in orienting downwards and descending from the pole suggesting significant motor impairments. On the other hand,

it was only the highest dose of JB that showed improvement in the ability of the animals treated with haloperidol to orient themselves downwards. Meanwhile, the doses of JB attenuated the impaired descending behavior induced by haloperidol. These findings suggest that JB at relevant doses might ameliorate motor incoordination produced by haloperidol in mice. The effects of JB on postural stability and fine motor coordination were further assessed in mice treated with haloperidol by challenging them to walk on the rod in the walk beam test. The distance traverse on the beam and the number of foot slips were monitored. Although haloperidol impaired movement in the walk beam test, it did not affect foot slips, which might be connected with muscle rigidity. Also, JB did not cause foot slips but it reverses the locomotor deficit produced by haloperidol on the walk beam at high doses. In the open field test, haloperidol in a similar characteristic manner, as reported in several studies, produced marked decrease in locomotion (Zhuravliova et al. 2007; Saeed et al. 2017; Sykes et al. 2017; Waku et al. 2021). Besides, haloperidol was also reported to produce abnormal movement such oral dyskinesia in animals, which was linked to morphological alterations of specific brain regions especially the striatum (Kelley et al. 1997; Halliday et al. 1999; Andreassen and Jorgensen 2000). However, JB at high doses improved the locomotor activity compromised by haloperidol in mice. Thus, the findings that JB reversed

motor impairment produced by haloperidol suggest its possible benefit as adjunctive remedy in mitigating motor disturbances in patients on prolonged haloperidol therapy.

Although haloperidol-induced parkinsonian-like syndromes have been ascribed to antagonism of nigrostriatal dopaminergic pathway, oxidative stress-mediated degeneration of dopaminergic neurons is known to play a prominent role in the decline of motor functions (Eyles et al. 1997; Sagara 1998; Post et al. 2002; Saeed et al. 2017; Saadullah et al. 2022; Saleem et al. 2022). Specifically, the neurotoxicity of haloperidol has been reported to be initiated by its cationic metabolites, which are transported by organic cation transporters to several brain regions such as substantia nigra, striatum, caudate nucleus and hippocampus (Eyles et al. 1997; Halliday et al. 1999; Post et al. 2002). In the dopaminergic neurons, these reactive metabolites inhibit mitochondrial complex I, thereby leading to induction of oxidative stress and neurodegeneration (Eyles et al. 1997; Post et al. 2002). Indeed, several studies have established increased activation of oxidative and nitric pathways as the main offenders of peroxidative neuronal cell death of the nigrostriatal dopaminergic and other neuronal pathways (Shivakumar and Ravindranath 1993; Halliday et al. 1999). In consonant with earlier studies, our data revealed that haloperidol increased whole brain MDA and nitrite concentrations, and promotes depletion of neuronal antioxidant molecules (GSH, catalase and SOD) suggesting activation of oxidative/nitric pathways as previously reported (Saadullah et al. 2022; Saleem et al. 2022). It has been reported that the parkinsonian-like symptoms only occur when dopaminergic neuronal cell death exceeds a critical threshold of about 70% of the striatal nerve terminals (Saeed et al. 2017). Several studies have also established increased susceptibility of dopaminergic neurons to oxidative stress, and inflammatory insults in haloperidol-induced motor dysfunctions (Post et al. 1998, 2002; Halliday et al. 1999; Saeed et al. 2017). Thus, oxidative stress-induced neuronal injury is further aggravated by the secondary release of inflammatory mediators suggesting the involvement of both oxidative and inflammatory pathways as co-conspirators in haloperidol-induced loss of dopaminergic neurons and onset of motor disorders (Saeed et al. 2017; Halliday et al. 1999; Post et al. 2002; Ben-Azu et al. 2018b, 2019). Although we did not measure the levels of oxidative stress markers after haloperidol treatment in discrete brain regions particularly in the striatum, which controls motor function, our data also support the previous findings of reduced antioxidant molecules, high concentrations of MDA/nitrite and proinflammatory cytokines, in the brains of mice treated with haloperidol (Ben-Azu et al. 2018a, 2018b). However, it is pertinent to note that oral administration of JB restores the deregulated levels of MDA/nitrite, antioxidant molecules, and

proinflammatory cytokines, which might be related to mitigation of the extrapyramidal adverse effects of haloperidol in mice.

The MAPK and CREB pathways have been implicated in the regulation of a variety of cellular functions such as proliferation, differentiation, survival, and death of neuronal cells, and are known to be affected by proinflammatory cytokines and cellular stressors such as oxidative stress (Wang et al. 2013; Chen et al. 2016; Mansour et al. 2018; Tong et al. 2018). It has been reported that deviation from the strict regulation of MAPK and CREB signaling pathway leads to the development of neurological disorders such as Alzheimer's disease, depression and PD (Wang et al. 2013; Mansour et al. 2018). The role of these signaling pathways in the neuropathology of PD has been further confirmed by the findings that rotenone, which is also an inhibitor of the mitochondrial complex I like haloperidol, activate microglial cells through the MAPK pathway and depletes CREB to cause dopaminergic neuronal damage in substantia nigra that ultimately results in parkinsonism (Chen et al. 2016; Mansour et al. 2018; Tong et al. 2018). More specifically, the response of neurons to activation of microglia promotes oxidative stress, neuroinflammation, and apoptosis, which eventually contribute to the death of dopaminergic neurons and progression of the disease (Chen et al. 2016; Mansour et al. 2018). Moreover, it has been reported that montelukast, which is a leukotriene receptor antagonist, exerted neuroprotective effects in the rotenone-induced PD animal model through the attenuation of MAPK activity (Mansour et al. 2018). In another study, minocycline was also shown to protect against rotenone-induced neurotoxicity via upregulation of CREB expressions (Sun et al. 2019). These findings strongly indicate that MAPK stimulation and downregulation of CREB contribute to rotenone's neurotoxicity in animal models of PD (Thomas et al. 2018; Sun et al. 2019). Incidentally, our data also showed increased activity of MAPK and reduced levels of CREB in brains of mice exposed to haloperidol, suggesting the likely involvement of these signaling pathways in haloperidol-induced neurotoxicity. But interestingly, mice that were treated with both JB and haloperidol had reduced MAPK and elevated CREB activity, indicating possible robust neuroprotection against dopaminergic neurodegeneration via downstream antioxidant/anti-inflammatory mechanisms.

Activation of MAPK activity and loss of neuroprotective function of CREB have been implicated in the increased oxidative-stress-mediated neuronal cell death that characterized the neuropathology of AD and PD (Mansour et al. 2018; Tong et al. 2018; Thomas et al. 2018; Sun et al. 2019; Behl et al. 1995). The deletion of CREB has been shown to cause non-specific neuronal cell death and progressive neurodegeneration in the hippocampus and striatum, which typified

neurodegenerative diseases (Tong et al. 2018; Saura and Carinaux 2017). The findings from this study further confirm that haloperidol causes pyknosis and atrophy of cytoarchitecture of the PFC, hippocampus and striatum of mice brains, as evidenced by the histological studies we performed. The histomorphometric studies showed that haloperidol caused neuronal cell loss and decreased population of viable neuronal cells in the PFC, hippocampus and striatum. The findings that these changes were associated with increased MAPK and reduced CREB suggest the possible involvement of these signaling molecules in haloperidol-induced loss of viable neuronal cells and motor dysfunctions in mice. Previous studies have also shown that histopathological changes and activation of transcriptional pathway that are associated with decreased neuronal cell viability occurred after chronic administration of haloperidol in experimental models (Post et al. 1998; Noh et al. 2000). However, in the intervention studies, high doses of JB attenuated haloperidol-induced loss of neuronal cells of the PFC, hippocampus and striatum of mice suggesting neuroprotection. It is pertinent to state that several studies have also reported that JB exhibited neuroprotective activity and preserved neuronal cells against oxidative and inflammatory insults in various animal models (Oyinbo et al. 2015; Umukoro et al. 2018a, b; Omorogbe et al. 2018). Unsurprisingly, HPLC investigations have shown that JB contains biologically active phytochemicals such as apigenidin, proapigenidin, apigenin, luteolin and naringenin (Geera et al. 2012; Benson et al., 2013; Makanjuola et al. 2016), many of which have been reported to exhibit various molecular mechanisms relevant for neuroprotection and anti-neuroinflammation functions (Lou et al. 2014; Li et al. 2016; Zhang et al. 2017; Umukoro et al. 2018a, b; Olugbemide et al. 2021). However, more studies are necessary to establish the roles of MAPK and CREB in the neuroprotective functions of JB against haloperidol-induced neurodegeneration and motor dysfunctions in mice.

Conclusion

The results of this investigation suggest that JB improves motor dysfunctions induced by haloperidol via neuroprotective mechanisms relating to antioxidant/anti-inflammatory and modulation of CREB and MAPK pathways, and may be of benefit as adjunctive remedy in reversing the parkinsonian-like adverse effects of haloperidol.

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Author contributions SU and JA designed the study; JA and AMA did the experiments; SU and BB did the analysis and wrote the draft of the manuscript. All authors read and approved the final version of the

manuscript.

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Data Availability The datasets generated during analyses of the experimental results related to the current study are available from the corresponding author on reasonable request.

Declarations

Competing Interests The authors have no relevant financial or non-financial interests to disclose.

Ethics approval The ethical principles established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8523, revised 2011) were followed.

Consent to participate Not applicable.

Consent to publish Not applicable.

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