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Research Article

Combination Antiretroviral Therapy (cART) induces CNS Neurotoxicity via Oxidative neuronal damage

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Abstract

Background: The newer approach to antiretroviral treatments using combination antiretroviral therapy (cART) has led to significant viral suppression but the associated adverse effects from the long-term exposure remain prevalent. This study evaluates the neurotoxicity and reproductive toxicity of cARTs. Methods: Behavioral studies using elevated plus maze, forced swim test, Morris water maze and open field test were used to assess neurobehavioral activities while biochemical parameters were assayed for brain oxidative markers. The reproductive hormones were analyzed along with seminal fluid. Histological staining of brain and testicular tissues was done to evaluate structural changes. Results: cART increased anxiety-related activities, induced memory deficit but showed no depressive activity. There was CNS-associated activity in the open field test, the brain antioxidant activity was reduced, and histological examination shows brain regions with cortical lesions. The reproductive hormones were unaltered and no histological abnormality was seen in the testes and epididymis. Conclusion: The outcome of the study showed that cART induces neurotoxic effect but no reproductive toxicity was observed.

Key Words: Neurotoxicity, combination antiretroviral therapy, HIV-Associated Neurocognitive Disorders, Morris Water Maze

INTRODUCTION

The discovery of antiretroviral drugs with significant efficacy against the human immunodeficiency virus (HIV) infection has been on the increase, however, monotherapy with these agents have not significantly reduced disease morbidity and mortality (Ariyo and Jones, 2022). Although each individual drug act via a different mechanism by which they are classified, the emergence of genotypic resistance is equally very common in antiretroviral monotherapy with attending adverse effect unique to each class (Zdanowicz, 2006). Hence, combining drugs of different classes and mechanistic targets has been recommended in antiretroviral therapy. Currently, none of the available antiretroviral drugs is curative but requires life-long therapy to achieve undetectable viremia, thus, a concerted adverse effect from prolonged exposure to combined therapy is likely as generally seen in polypharmacy (Massawe *et al*., 2023).

Utilization of a combination antiretroviral therapy (cART) approach in HIV management has caused the disease to become a chronic manageable disease, there is reduced occurrence of genotypic drug resistance and controlled viral load but the attending challenge has been certain exacerbated adverse effects which were a serious concern (Crowell *et al*., 2014). Although there are numerous classes of antiretrovirals, cART is essentially a combination of two nucleoside reverse transcriptase inhibitors (NRTIs) and an integrase inhibitor (Rai *et al*., 2018). Superiority studies showed that in terms of efficacy, Lamivudine and Tenofovir were model NRTIs of choice while Dolutegravir is the ideal Integrase Inhibitor of choice in cARTs. These individual drugs of choice in cART also have tolerable adverse effects when compared to other members of their respective classes (Dalakas, 2001). The Lamivudine-Tenofovir-Dolutegravir cART is a commonly prescribed first-line antiretroviral agent and it is largely well tolerated. However, besides these positive effects, many patients who are on therapy experience drug toxicities that include neurological and reproductive complications (Awodele *et al*., 2018). Studies have suggested that the reproductive complication may not be a result of cART-induced toxicity but a result of co-morbid infections like Herpes simplex in people living with HIV (Brenda *et al*., 2015). HIV-Associated Neurocognitive Disorders (HAND) is a common neuropsychiatric phenotype seen in patients with HIV, characterized by cognitive decline and anxiety-related disorders (Gibbie *et al*., 2006). In the pathophysiology of HIV

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infection, the virus triggers inflammatory responses in the brain causing the commonly seen neuronal dysfunction amongst patients living with HIV and an argument has been raised whether the persisting neurological complication is a result of the viral infection itself or from the adverse effect of long-term cART exposure (Brew *et al*., 2009). The later, that the mechanisms contributing to the persistent neuroinflammation may be cART-induced was hypothesized to be true from a cohort study of patients living with HIV which shows persistent HIV-Associated Neurocognitive Disorders (HAND) despite reduced viral load (Underwood *et al*., 2015).

Furthermore, improved cognitive function sequel to discontinuation of cARTs in patients with suppressed viremia has been reported (Yuan and Kaul, 2021). In order to elucidate the underlying mechanism, CNS penetration effectiveness (CPE) rank score was proposed, but findings show that CPE score was not significantly related to cognitive functioning neither was there a disparity in improved neuropsychological outcomes in patients on antiretrovirals with a high or low degree of CPE; suggesting that alternate mechanisms might be responsible for cARTs neurological toxicity (Yuan *et al*., 2021). Several studies have suggested neuroinflammation as a consequence of the adverse effects at the cellular level of cART, independent of HIV since at least some ARV compounds may themselves have neurotoxic effects (Akay *et al*., 2014). The objective of the study is thus to elucidate the neurotoxic effects of cARTs and identify underlying pathogenesis.

MATERIALS AND METHODS

Test Compound: The test compound is a combined antiretroviral drug containing 300 mg Tenofovir disoproxil fumarate, 300 mg Lamivudine and 50 mg Dolutegravir per tablet. The drug was purchased from a pharmacy store in Lagos State, Nigeria.

Animals: Approximately 40 male and 40 female rats weighing 60 - 70 g were used in the experimental protocol. Animal handling was as approved by the University of Ibadan Animal Care and Use Research Ethics Committee, UI-ACUREC (Ethical no: UI-ACUREC/016-0120/29). The rats were randomly grouped into four treatment groups (n = 10), administered 10 mL/kg distilled water and 2, 5 and 10 mg/kg cART orally by means of a polythene cannula and animals received their doses once a day for ninety days.

Anxio-depressive related behaviors: Behavioral phenotyping was performed using Elevated Plus Maze (EPM) for assessment of anxiety–like behavior, Forced Swim Test (FST) for assessment of depressive-like phenotype, Moris Water Maze (MWM) test to assess memory function and open field test to rule out unspecific locomotor activity.

Elevated Plus Maze: The elevated plus maze is composed of a pair of open (50 × 10 cm) and closed arms (50 × 10 ×50 cm) facing one another, elevated to about 55 cm height. The arms are connected to one another at the center area (10 × 10 cm) which is also open. The closed arms are darkened by painting the covers black. Each rat was placed at the open arm facing outward and observed for 5 minutes. The number of entries into the open arms and closed arms was recorded, as well as the time spent in the respective arms during the 5 minutes duration. Entry into an arm is when all four limbs are in the particular arm of the maze. The apparatus was cleaned after each animal session using 70% ethanol to remove the cue of the previous animal. Results were expressed as the mean of the number of entries and time spent in open and closed arms for each treatment group and the index of open-arm avoidance was calculated as the ratio of total time and entries by rats (Pellow and File, 1986).

Forced Swimming Test: Animals were placed to swim in an open cylindrical container with a diameter (12 cm) filled up to 43 cm mark with water (22 ºC). The period of immobility was measured for 4 minutes after an initial 2 minutes of acclimatization during which immobility is not recorded as by Porsolt (Can *et al*., 2012). Immobility is when the rat is not struggling and just floats and makes movement necessary to keep afloat (head above water). Time sampling techniques were used to separate the different active behaviors (struggling, climbing) in water.

Morris Water Maze Test: Morris water maze is a circular pool of diameter 120 cm and height of 30 cm filled with milky (opaque) water enough to cover a submerged (hidden platform) circular (diameter 12 cm) escape platform by 1cm (Vorhees and Williams, 2006). The circular pool was divided into 4 quadrants (north, east, west and south) with the hidden platform placed in a specific spot in a particular quadrant. Each rat was dropped into the quadrant opposite the quadrant with the hidden platform facing the circumference of the pool. The rat was allowed to swim to safety on the hidden platform and time taken was recorded. Rats that could not find the platform in sixty seconds were guided to the platform and allowed to stay on the platform for about 1-2 minutes. The procedure was repeated 3 times per day for each animal for 3 consecutive days during trials. Twenty-four hours after the last trial (fifth day), each rat was allowed a single probe trial with the platform removed. The time spent in the target quadrant, number of times the animal crossed the spot platform was initially located and average speed in target quadrant were recorded.

Open Field Test: The test animals were placed individually in the center of the open field box and were allowed to explore for thirty minutes. During the last ten minutes, novelty induced behaviors (rearing and grooming), latency to leave the starting square and the total number of lines crossed were recorded (Oyekunle *et al*., 2010). The box was cleaned with 70% ethanol after each animal exploration to remove cues.

Tissue Harvesting and Analysis: For the reproductive hormonal assay, blood samples were collected through cardiac puncture into non-heparinized bottles and centrifuged at 3000 rpm for 15 minutes. The serum was separated from the cells into another plain serum bottle using micro pipette and stored in icepacks. For biochemical and histological assessment, animals were anaesthetized and sacrificed. The brain and testes were excised and placed in 10% buffered formalin. Seminal analysis, sperm progressive motility (%), was done immediately. The cauda epididymis was cut open and semen was squeezed on a clean microscope slide. Two drops of warm 2.9% sodium citrate was added to the semen sample, covered with cover slip and examined under a light microscope using x 40 power objective lens magnification.

Reproductive Hormone Assay: The hormonal assay for serum concentration of testosterone, luteinizing hormone (LH), follicle stimulating hormone (FSH) and estradiol (E2) was done using commercially available Enzyme linked immunosorbent assay (ELISA) kits.

Biochemical Assay: Excised brain tissues were homogenized and centrifuged. The supernatant which contains mitochondrial fraction was assayed for brain malondialdehyde (MDA) concentration, superoxide dismutase (SOD) levels, glutathione (GSH) concentration and catalase activity using spectrophotometric method.

Histological studies: The fixed brains and testicular tissues were processed in an automatic tissue processor (LEICA TP1020), followed by embedding, microtomy, floating, drying, and staining with Haematoxylin and Eosin (H&E). The prepared slides were examined at 400x magnification with an Olympus CH (japan) binocular microscope for histomorphology changes in the brain, testes, and epididymis.

Statistical Analysis: Results were expressed as mean ± SEM. Statistical analysis of data was done using one-way ANOVA, followed by Tukey post- hoc test for multiple comparison among the groups. The data were analyzed using Graph pad prism software version 7.00. p - values less than 0.05 ($p < 0.05$) was considered statistically significant.

RESULTS

Effect of cART on anxiety-like behavior in Elevated plus maze: Long -term treatment with cART significantly increased total time spent in close arm in a dose dependent manner. However, a significant decrease was noticed only in rats that received 8 mg/kg of cART ($p<0.05$), compared with the control group. Also, there was significant increase in time spent in open arm at dose of 8 mg/kg as compared to control (Figure 1A). The percentage alternation in arm entries behavior was significantly reduced ($p<0.05$) in rats treated with cART at doses 4 and 8 mg/kg as compared to control group (Figure 1B).

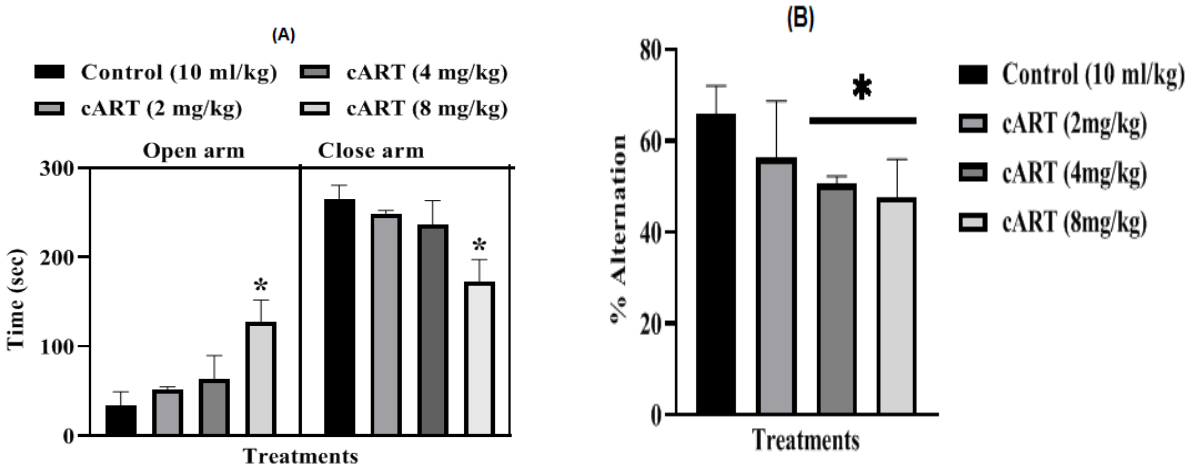


Figure 1:
The effects of different doses (2, 4 and 8 mg/kg) of cART on (A) Total time spent in open arm and close arm, (B) percentage alternation in arm entries in the elevated plus-maze test. Results are expressed as mean ± SEM (n=10). Oneway ANOVA followed by Tukey's post- hoc test, **p*<0.05 vs. control.

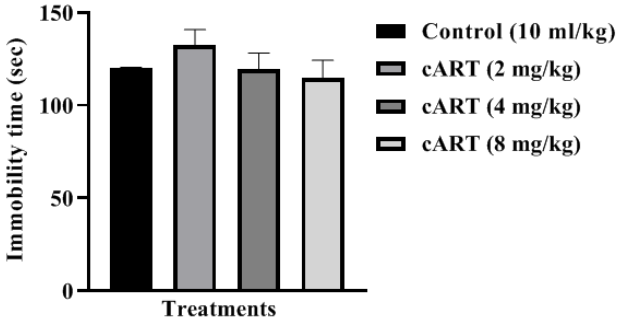


Figure 2:
The effects of different doses (2, 4 and 8 mg/kg) of cART on immobility time in forced swim test. Results are expressed as mean ± SEM (n=10). One way ANOVA followed by Tukey's post- hoc test.

Effect of cART on immobility time in Forced Swim Test: The effect of cART on forced swim test is shown in Figure 2. Tukey post hoc test revealed that rats treated with cART (2, 4, 8 mg/kg) showed no significant difference in immobility time compared to the control.

Effect of cART on Morris Water maze Test: The result of Morris water maze test revealed that cART significantly increased ($p <0.05$) escape latency time in rats in the treatment groups when compared to control group (Figure 3A). Effect of cART on annulus time measured as time spent in target quadrant was significantly reduced ($p <0.05$) as compared to control group (Figure 3B).

Effect of cART on anxiety-like activity in Open Field Test: There was a significant increase in the number of line crossing in cART treated groups when compared to the control group (Figure 4A). Also, novelty induced rearing and grooming activities were significantly increased in groups treated with 4 and 8 mg/kg cART (Figure 4B and 4C).

Effect of cART on neurooxidative biochemical markers: Long term treatment with cART showed no significant difference in MDA levels as compared to control group (Figure 5A). However, SOD level was significantly decreased ($p<0.05$) in rats administered doses 4 and 8 mg/kg cART as

compared to control (Figure 5B). cART at all administered doses significantly decrease catalase and GSH levels as compared to vehicle controls (Figure 5C and 5D respectively).

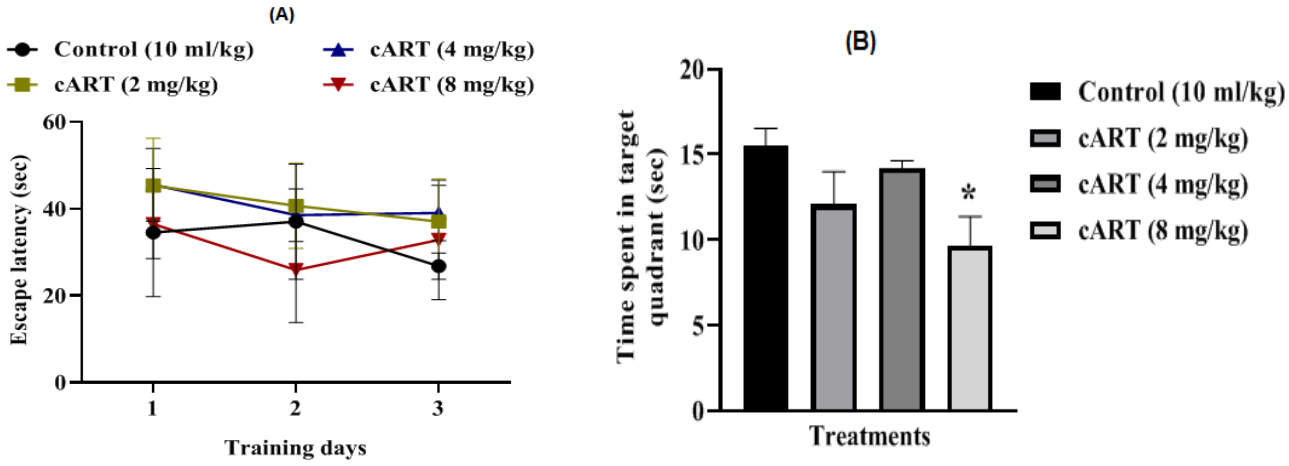


Figure 3:
The effects of different doses (2, 4 and 8 mg/kg) of cART on (A) Escape latency, (B) time spent in target quadrant in Morris water-maze test. Results are expressed as mean ± SEM (n=10). One-way ANOVA followed by Tukey's post- hoc test, **p*<0.05 vs. control.

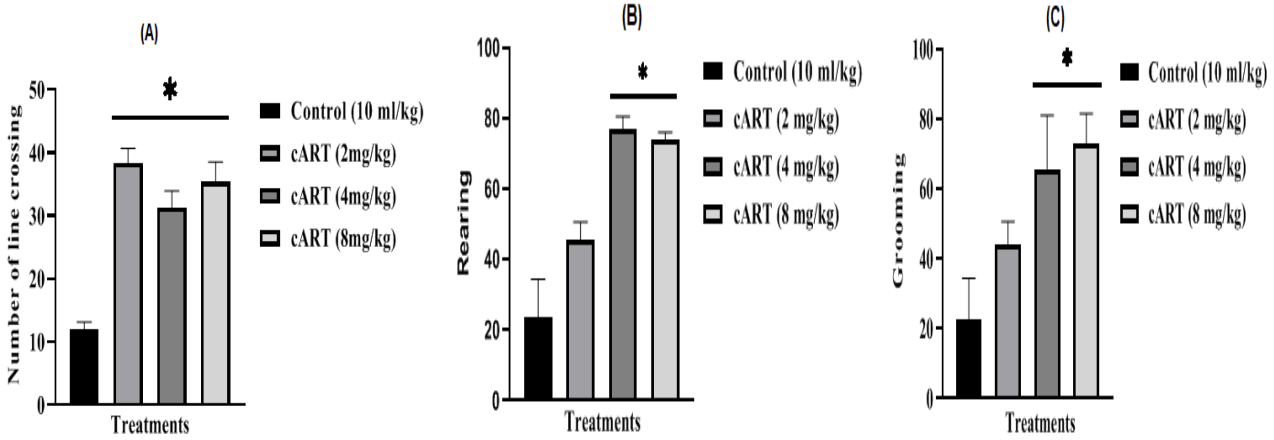
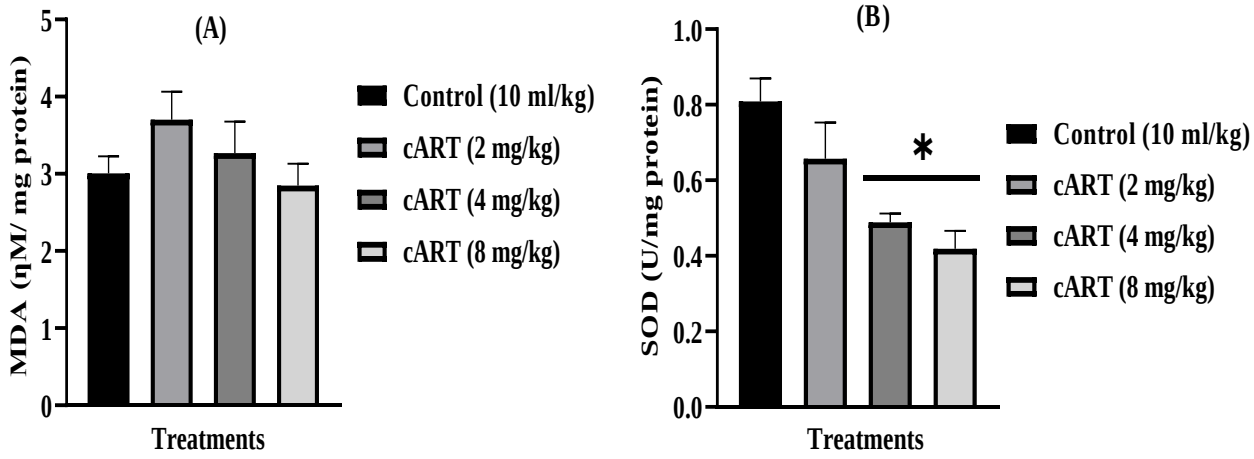


Figure 4:
The effects of different doses (2, 4 and 8 mg/kg) of cART on (A) Locomotor activity, (B) Novelty induced rearing (C) Novelty induced grooming in open field test. Results are expressed as mean ± SEM (n=10). One way ANOVA followed by Tukey's post- hoc test, **p*< 0.05 vs. control.



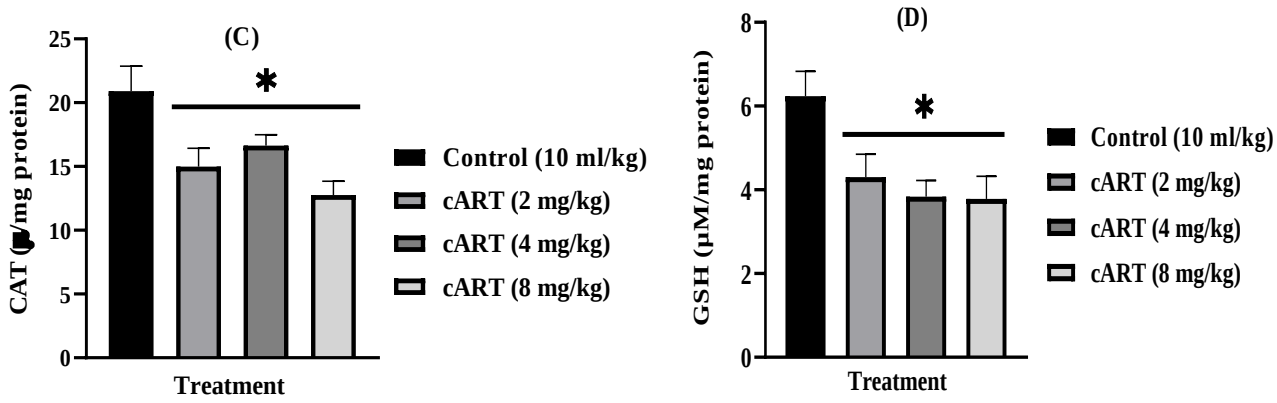


Figure 5:
The effects of different doses (2, 4 and 8 mg/kg) of cART on (A) MDA levels, (B) SOD levels, (C) Catalase levels, (D) GSH levels. Results are expressed as mean ± SEM (n=10). One way ANOVA followed by Tukey's post- hoc test, **p*<0.05 vs. control

Table 1:
Effect of cART on reproductive hormones

Animal Sex	Parameters (Plasma)	Control (10 mL/kg)	cART (2 mg/kg)	cART (4 mg/kg)	cART (8 mg/kg)
Female	**FSH (mlU/mL)**	1.63±0.10	1.95±0.10	1.68±0.05	1.80±0.08
	LH (mlU/mL)	0.87±0.08	0.94±0.04	0.78±0.05	0.89±0.05
	P (ηg/mL)	34.6±2.17	39.8±4.41	43.4±2.40	40.1±3.96
	E (ρg/mL)	34.5±2.10	33.9±3.84	39.4±4.48	36.90±4.04
Male	**FSH (mlU/mL)**	1.75±0.09	2.01±0.18	1.92±0.11	1.78±0.18
	LH (mlU/mL)	0.713±0.04	0.87±0.02*****	0.85±0.03*****	0.85±0.04*****
	T (ηg/mL)	0.16±0.01	0.34±0.02	0.27±0.01	0.42±0.01

*Values are presented as mean ± SEM * significantly different from the control group at p≤0.05. FSH: follicle stimulating hormone, LH: luteinizing hormone, P: progesterone, E: estradiol and T: testosterone.*
Results are expressed as mean ± SEM (n=10). One way ANOVA followed by Tukey's post- hoc test, **p*<0.05 vs. control.

Effect of cART on reproductive hormones
Long term treatment with cART showed no significant difference in hormones levels as compared to control group. However, LH level was significantly increased (p<0.05) in male rats in all treatment groups when compared to the control group (Table 1).

Effect of cART on sperm count, viability and motility:
The result of seminal fluid analysis showed no significant difference in sperm count of cART-treated rats compared to the vehicle-treated control (Figure 6A). Long-term administration of cART at the dose of 8 mg/kg significantly reduced (p<0.05) sperm viability and sperm motility compared to control (Figure 3A). However, doses 2 mg/kg and 4 mg/kg showed no significant difference in sperm viability and sperm motility compared to control (Figure 6B and Figure 6C respectively).

Effect of chronic administration of cART on histomorphology of Cerebellum and Prefrontal cortex:
The results of H&E staining examination of brain sections, cerebellum and prefrontal cortex (Figures 78 respectively), showed pyknotic neurons in cART-treated animals as compared to vehicle-treated animals.

Effect of chronic administration of cART on histomorphology of Testes and Epididymis:
The results of H&E staining examination of the testes and epididymis (Figure 9 and10 respectively) showed no significant morphological difference in tissue cytoarchitecture of cART-treated animals as compared to vehicle-treated animals.

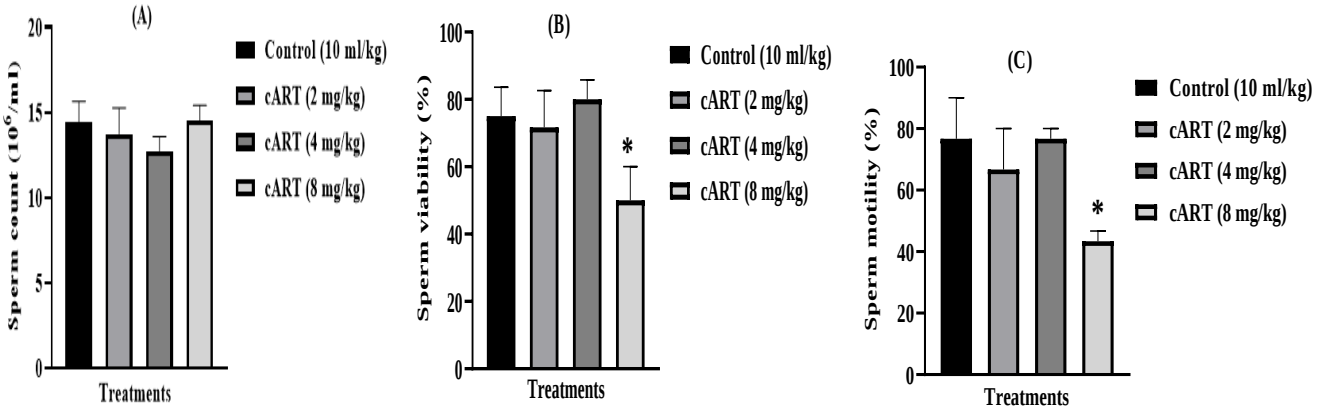


Figure 6:
The effects of different doses (2, 4 and 8 mg/kg) of cART on (A) Sperm count, (B) Sperm viability, (C) Sperm motility. Results are expressed as mean ± SEM (n=10). One-way ANOVA followed by Tukey's post- hoc test, *p<0.05 vs. control.

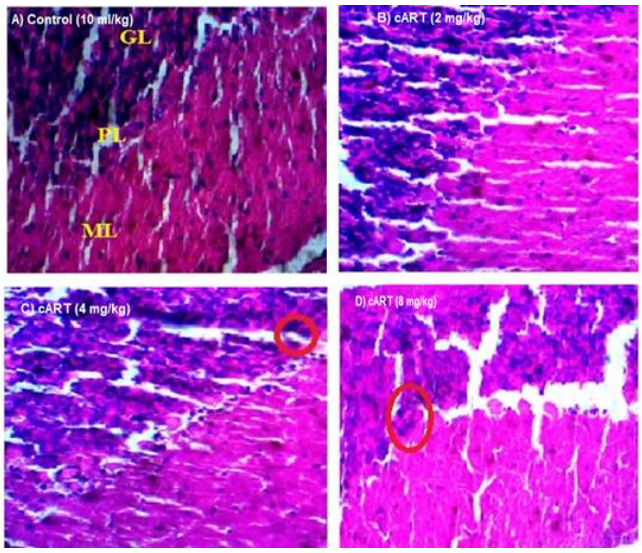


Figure 7:
Hematoxylin and Eosin-stained photomicrograph sections of the cerebellum. GL: granular layer; PL: purkinje layer; ML: molecular layer; red circle: pyknotic cells; Magnification: ×400

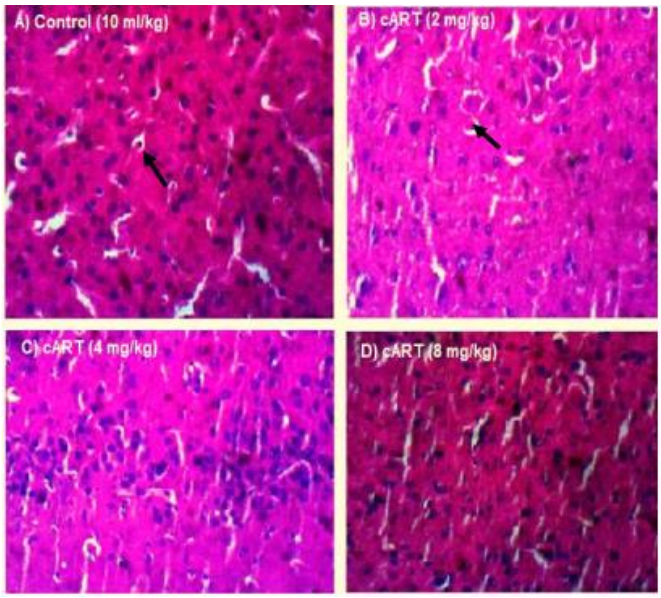


Figure 8:
Hematoxylin and Eosin-stained photomicrograph of sections of the prefrontal cortex showing pyknotic cells in all the groups. Magnification: ×400

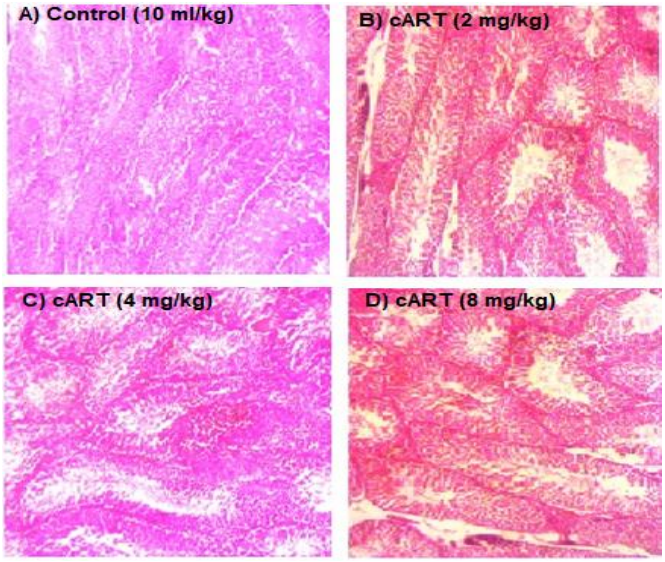


Figure 9:
Hematoxylin and Eosin-stained photomicrograph sections of the testes. Magnification: ×400

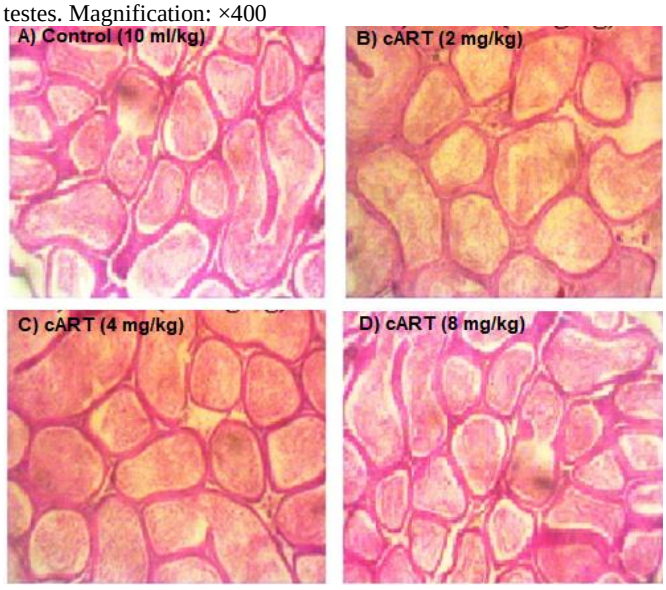


Figure 10:
Hematyoxylin and Eosin-stained photomicrograph sections of the epididymis. Magnification: ×400

DISCUSSION

The pattern of neurologic side effects in patients living with HIV pre and post-cART era are significantly distinct, and if introduction of cART is the attending variable, it is likely that cART is responsible for these distinct side effects seen as HAND commonly characterized by anxiogenic and depressive state, memory deficit and impairment of executive function in patients on cART regimen despite controlled viremia (Heaton *et al*., 2011).

Anxiety is a defined neurobehavioral disorder that may be the "state" which is due to some external stimuli or "trait" which is innate (Steimer, 2002) and the possibility of cART precipitating anxiety state was evaluated. In the elevated plus maze (EPM) test, an animal model for predictive validity, long-term exposure to cART increased anxiety-like behavior

in the treated group suggesting its anxiogenic effect. The time spent in open arm by cART-treated animals was significantly reduced while time spent in closed arm was prolonged and this clearly suggests that cART precipitates anxiety behavior.

The hallmark of depression is behavioral despair which can be measured with the forced swimming test, where animal degree of immobility after inescapable state correlates to measure of depressive behavior (Kaufman, 2006). In the FST, long-term exposure to cART did not alter the degree of immobility in animals and there was no significant difference in the immobility time (which may suggest a depressive-like behavior) in the treatment groups when compared to the control group. Thus, cART may not induce depression; however, depression itself has been defined as a heterogenic disorder with diverse etiology whose pathophysiology is not completely clear (Buch *et al*., 2021). Hence, the depressive behavior seen in patients on cART regimen may have other underlying causes and these cannot be said to be completely independent of the drug exposure.

Spatial memory was assessed by the Morris Water Maze test which consist of a three-days training period (visible and invisible platform training sessions) and a probe trial on the fifth day. The cognitive test was assessed in the Moris Water Maze test (Bhutada *et al*., 2010). The mean escape latency of the trained rat decreased over the course of trial in all treatment groups compared to the control and the time spent in target quadrant decreased across the group suggesting that cART caused learning impairment and decrease in cognitive behavior compared to the control group in annulus time and crossing.

In this study, the CNS effect of cART was examined for rearing, grooming and locomotor activity in a novel environment using open field test. Rearing, which involves the animal standing on its hind legs and raising its front paw on air or the walls of the maze is a measure of exploratory behavior and is considered to be central excitatory behavior (Brown *et al*., 1999). Long-term treatment with cART produced an increased rearing behavior in the animals, suggesting that it might have central nervous system excitatory activity. Grooming in animals plays a deactivating role in restoring homeostasis under stressful conditions with drowsily effect of the central nervous system (Aderibigbe *et al*., 2010). The increased grooming among cART-treated animals as observed in this study therefore suggests an excitatory effect of cART on the CNS. Locomotion in animals like rearing is indicative of their exploratory behavior and it is considered to be a central excitatory behavior (Ajayi and Ukpomwan, 1994). An increase in locomotory activity or hyperactivity-like behavior was observed in animals in cART-treated groups when compared to the control group. The CNS is central to higher cognitive function and the CNS effect of cART therefore suggests that it plays a critical role in the impairment of executive function commonly seen in patients on cART regimen.

CNS impairment is commonly associated with dispersed cortical lesions resulting from oxidative stress-induced cell damage (Zhang *et al*., 2014). Oxidative stress is caused by an imbalance between the production of reactive oxygen and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage (Benhar *et al*., 2002). In this study, long-term exposure to cART decreased brain antioxidant enzymes seen as a significant decrease in glutathione level, superoxide dismutase and catalase levels. However, cART did not precipitate a prooxidative effect as there was no significant difference in MDA levels in cART treated animals when compared to the vehicle-treated controls. Therefore, the neurological toxicity of cART is from its effect on the decrease in the level of biologic antioxidant enzymes.

Histological evidence also shows areas of necrosis in the cerebellum which suggests cART-induced oxidative damage as an underlying cause of HAND side effects seen in patients. Generally, cerebellar lesions are characterized by motor dysfunctions, however, recent findings have identified attending neuropsychological deficits described as Cerebellar Cognitive Affective Syndrome (CCAS). The syndrome is marked by impairment in four areas of cognitive functioning; the executive functions and attention, memory and visuospatial functions, language, and emotions (Schmahmann & Sherman, 1998). In a study using logistic regression models and sub-models, the results suggested that a condensed battery of neurobehavioral tests can equally be used to detect CCAS (Bolceková *et al*., 2017). Although the definitive pathophysiology of CCAS is still under study, the associated cognitive derangement has clearly been established (Tedesco *et al*., 2011). Similarly, findings in this study showed associated behavioral decline with attending histological lesions in the cerebellum.

Reproductive toxicity of cART was also assessed by analyzing the effects of cART on key reproductive hormones (luteinizing hormone, follicle-stimulating hormone estradiol, and testosterone). Results from the study shows no relationship between cART and reproductive hormonal change.

Although the level of LH increases in treated male wister rats compared to the control, this has no impact on the overall reproductive capability of the animals as the testosterone level was not affected (Oduwole *et al*., 2021). On seminal fluid analysis, cART caused decrease in the motility of the sperm but there was no significant difference in sperm count and viability when compared to the control group.

The histopathology of the testes and epididymis as evidence of reproductive toxicity shows no significant changes in the testes and epididymis of cART-treated animals when compared to the control.

CONCLUSION

The combined antiretroviral therapy may exhibit characteristic neurotoxic adverse effects evidenced by the oxidative stress-induced neuronal damage and cART-induced lesion in the cerebellum. However, there might be no associated reproductive toxicity mediated based on the results obtained from the reproductive parameters evaluated in this study.

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