Highly active antiretroviral therapy-silver nanoparticle conjugate interacts with neuronal and glial cells and alleviates anxiety-like behaviour in streptozotocin-induced diabetic rats

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Abstract

The inception of highly active antiretroviral therapy (HAART) has changed the management of human immunodeficiency virus (HIV) positive patients, and their life expectancy has improved. However, neurological complications associated with chronic HAART administration has not been fully addressed. Therefore, this study evaluated the potential benefits of silver nanoparticles (AgNPs) conjugated HAART and its interaction with neuronal and glial cells in type-2 diabetic rats. Forty-two (42) adult male Sprague-Dawley rats (250 ± 13 g) were divided into non-diabetic and diabetic groups. After induction of diabetes, non-diabetic and diabetic animals were administered either with de-ionized water (DW), HAART (98.2 mg/kg, p.o) or AgNPs + HAART (24.5 mg/kg, i. p) for eight weeks. After that, metabolic biomarkers, oxidative injury, tissue inflammation, and animal behavioural changes were evaluated. Also, the prefrontal cortex was excised for immunohistochemical and ultrastructural analysis. The HAART-treated diabetic rats showed a significant increase in blood glucose level, number of faecal pellets, malondialdehyde (MDA), and pro-inflammatory cytokines (TNF-α, IL-1β) while locomotion, reduced glutathione (GSH), superoxide dismutase (SOD) activity, and PFC-GFAP positive cells were significantly reduced compared with diabetic control. However, administration of AgNPs + HAART to diabetic rats significantly improved the blood glucose level, metabolic activities, SOD, GSH, PFC-GFAP positive cells, protecting neuronal injury while reducing MDA and anxiety-like behaviour in the open field test. Administration of HAART aggravates anxiety-like behaviours and promotes neurotoxic effects in the PFC of diabetic rats. However, AgNPs + HAART alleviates the anxiogenic effects of HAART, and preserves PFC GFAP-positive cells and neuronal cytoarchitecture by reducing oxidative and neuroinflammatory injury.

Introduction

The introduction of highly active antiretroviral therapy (HAART) has changed the Human Immunodeficiency Virus (HIV) diagnosis from a fatal disease into a chronically managed condition. Consequently, the life expectancy and the quality of life among the people living with HIV has since improved (Lewden et al., 2012). Despite the benefits of HAART, its long-term use and systemic exposure has been strongly linked with various metabolic disturbances such as diabetes and cardiovascular diseases (Nansseu et al., 2018). Previous studies have reported that initiation of HAART increases the risk of diabetes mellitus (Ergin et al., 2020; Nansseu et al., 2018). The mechanism by which HAART causes diabetes has been attributed to excessive reactive oxygen species (ROS) production, leading to cell toxicity (Hulgan et al., 2003; Nsonwu-Anyanwu et al., 2017; Sharma, 2014). The increased ROS damage vital cellular components (e.g., DNA, lipids, and proteins) in glucoregulatory tissues leading to insulin resistance or compromised insulin synthesis, thereby promoting hyperglycemia (Han, 2016). Also, HAART has been linked with mitochondrial damage and subsequent increase in the risk of neuropathy and neuroinflammation (Lin et al., 2018; Lucas et al., 2011). Chronic administration of HAART to HIV positive patients has been reported to cause neuroinflammation, changes in astrocyte mitochondrial membrane and mitochondrial ROS production (Anthony et al., 2005; Cohen et al., 2017).

The astrocytes are the major components of the brain tissue involved in the overall maintenance of brain homeostasis, neuronal metabolism and neuroprotection (Siracusa et al., 2019). Thus, active astrocyte dysfunction during hyperglycemia characterized by a decrease in glial fibrillary acidic protein (GFAP) level has been reported to promote neurocognitive dysfunctions (Coleman et al., 2004; Yang et al., 2018; Zhang et al., 2015).

The continuous use of HAART to prevent a viral rebound in people living with HIV and diabetes-induced neuroinflammation, caused detrimental effects on astrocytes in the CNS and contributed significantly to the aetiology of neuro-pathologies (Cohen et al., 2017; Yang et al., 2018). Excessive production of pro-inflammatory cytokines during neuroinflammation have been implicated in cognitive deficits and anxiety disorders (Charlton et al., 2018; Li et al., 2019). Interestingly, in the post-era of HAART, people living with HIV have experienced an improvement in motor skills and verbal fluency but show impaired executive functions and anxiety-like behaviour (Checa et al., 2020; Heaton et al., 2011). In addition, the prevalence of anxiety and depression among the patient receiving HAART remains high (Nuesch et al., 2009; Rabkin et al., 2000). The most used components of HAART (Efavirenz and Tenofovir) have been reported to cross the blood-brain barrier, causing mitochondrial dysfunction and some neurological-related adverse effects like depression and anxiety disorder (Checa et al., 2020; Chen et al., 2019). Several studies have suggested that the prefrontal cortex and its circuitry play a vital role in anxiety-like behaviour in animals and humans (Hare & Duman, 2020; Likhtik et al., 2014). More so, a decrease in the prefrontal cortex activities and abnormalities in the neuroimaging studies have been observed in fearful and anxious individuals (Berkowitz et al., 2007; Likhtik et al., 2014).

The application of nanomedicine for antiretroviral drugs delivery holds promise in HIV therapeutics due to their unique advantages such as increased drug bioavailability, stability, ability to reach the target cell population, and half-life (Kumar et al., 2015).

The primary issue with HAART is that it requires high doses for a prolonged duration of time to reduce the viral level in the system, thus predisposing living tissue to toxicity (Kumar, 2019).

Silver nanoparticles (AgNPs) exhibit novel properties, making them suitable for a wide range of applications in the biomedical field. In addition, AgNPs are the most-studied and utilized nanoparticles due to their simple method of synthesis, high surface to volume ratio, unique morphology and intracellular delivery system (Marin et al., 2015). AgNPs has been utilized as antiviral, antidiabetic and antioxidant agents in the biomedical field (Vadlapudi & Amanchy, 2017).

Conversely, in vitro and in vivo studies on the neurotoxic effects of silver and silver nanoparticles reported a size- and dose-dependent cellular uptake and toxicity (Ferdous & Nemmar, 2020; Greish et al., 2019a). Small-medium-sized nanoparticles have been reported to be less toxic to the cell (Lara et al., 2010). More so, studies have reported that cytotoxic effects of silver nanoparticles can be minimized by reducing silver ions to a ground state (from Ag^{+} to Ag^{0}), synthesizing a spherical shape, small-medium size, and

modified surface area (Dlugosz et al., 2020; Smith et al., 2018). Another study suggests that the cytotoxic effect observed in the use of silver nanoparticles is due to silver ions exposure (Wang et al., 2014).

However, there is no data to substantiate the interaction of HAART conjugated with silver nanoparticles on neuronal cells and neurocognitive dysfunctions. Hence, this study assessed the role of HAART-silver nanoparticles conjugate on the PFC of STZ-induced diabetic rats.

Materials And Methods

Materials

The Atripla, a combined form of Efavirenz (EFV, 600 mg), Emtricitabine (FTC, 200 mg) and Tenofovir disoproxil fumarate (TDF, 300 mg), was purchased from Dis-Chem pharmacy Ballito, South Africa. Streptozotocin (STZ), trisodium citrate, Sodium hydroxide and silver nitrate ($AgN0_3$) of analytical grade were sourced from Sigma-Aldrich Company, Johannesburg, South Africa. Enzyme-linked immunoassay (ELISA) kits for TNF-α (E-EL-R0019), and interleukin (IL)-1β (E-EL-R0012) were purchased from BIOCOM Africa (pty), Ltd, South Africa. All the chemicals, reagents, and equipment were of analytical grade.

Experimental animal

Forty-two (42) adult male Sprague-Dawley rats (250 ± 13 g) were obtained from the Biomedical Research Unit (BRU) of the University of KwaZulu-Natal and were housed in the standard animal laboratory room. The animal laboratory room was maintained at a temperature of 24 - 26 $^{\circ}$C, 12:12 light: dark cycle and 40-60% humidity. The animals were allowed free access to water and feed *ad libitum*. All animals were handled according to the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996. The animal laboratory procedures were approved by the Animal Ethics Committee of the University of KwaZulu-Natal (AREC/044/019D).

Experimental design

After acclimatization for six (6) days, the rats were randomly divided into six groups (n=7 per group) and were treated for eight weeks, as in figure 1. Drug dosage adopted was according to (Everson et al., 2018).

Induction of Type II Diabetes in rats

Experimental type 2 diabetes mellitus was induced using a fructose-streptozotocin (STZ) rat model as described by (Wilson & Islam, 2012). Briefly, rats received 10% fructose solution *ad libitum* for two weeks. After that, the rats were fasted overnight and injected with a single of 40 mg/Kg B.W. STZ i.p. The STZ was dissolved in 0.9% NaCl with 100 mM sodium citrate buffer (pH 4.5). The control rats received an

equal volume of the buffer. Animals with fasting blood glucose levels ≥ 200 mg/dL were considered diabetic and included in this study.

Formulation of AgNPs + HAART

Silver nanoparticles were synthesized according to Turkevich et al. (Turkevich et al., 1951). Briefly, an aqueous solution (0.03 M) of silver nitrate ($AgNO_3$) was prepared from 5.10 g of $AgNO_3$ crystal. Then, a stock aqueous solution (2 M) of trisodium citrate (TSC) was prepared from 147 g in 250 mL of double-distilled water and used as a reducing and stabilizing agent. Four TSC solutions with varying concentrations (0.5M, 1M, 1.5M & 2M) were prepared to test the $AgNO_3$ stability.

The HAART silver nanoparticle (AgNPs + HAART) was prepared by dissolving 15 g of HAART in 10 mL of concentrated sodium hydroxide solution, and distilled water was added to make 50 mL. The final concentration for this mixture was 1.0452 of HAART, and this mixture was stirred on ultra-sonication to ensure proper reaction of HAART and AgNPs.

The AgNPs + HAART was centrifuged at 4,500 rpm and 40 °C for 40 minutes to discrete the unincorporated drug. The supernatant was analyzed using a UV spectrophotometer at a wavelength of 285-315 nm to calculate the quantity of unincorporated drug (W1) from the total amount of drug coupled with silver nanoparticle (W2).

The AgNPs + HAART percentage incorporated efficiency was calculated according to Govender et al. (Govender et al., 2006) as follows:

$$\% IE = \frac{W_2 - W_1}{W_1} \times 100 = 90.52 \pm 0.5\ \%.$$

Characterization of AgNPs and HAART + AgNPs

The characterization of AgNPs and AgNPs + HAART was previously done (Lawal et al., 2021). Briefly, Fourier Transform Infrared (FTIR) spectroscopy (Perkin-Elmer Universal ATR spectrometer, USA) was used to identify the various functional groups in the HAART + AgNPs conjugates. The HAART and AgNPs show absorption bands at 3303.13 cm^{-1} and 3227.27 respectively in the FTIR spectrum.

The ultraviolet-visible (UV-Vis) spectroscopy (Shimadzu MultSpec-1501, Shimadzu Corporation, Tokyo, Japan) indicates that HAART + AgNPs synthesized with 1.5M AgNPs possess a significant smaller nanoparticle than the other concentrations.

The size and morphology of the nanoparticles were examined by a high-resolution transmission electron microscope (HR-TEM, JEOL 2100, Japan) operated at a voltage of 200 kV. The nanoparticles (NPs) size for the synthesized HAART + AgNPs was 30-50 nm, while morphology was spherical.

The field emission scanning electron microscope (FESEM, Carl Zeiss, Germany) operated at a voltage of 5 kV with energy dispersive X-ray (EDX, Aztec Analysis Software, England) was used to determine the elemental components. The presence of silver, sodium and some functional groups indicated the silver nanoparticles were successfully incorporated with HAART.

Blood glucose level and metabolic activities

The weekly fasting blood glucose was determined using a portable glucometer (Sigma-Aldrich, Durban, South Africa), and the blood sample was obtained through the tail vein. The metabolic activities (characterized by calorie intake, water intake, urine volume and the number of faecal pellets) were monitored in individual rats using a novel metabolic cage.

Behavioural assessment

Open field test (OFT) to measure the anxiety and explorative behaviours

On day 79 of the experiment, the animals were evaluated for spontaneous and anxiety-like behaviours using the open field test. The open field apparatus consists of a large rectangular box measuring 70cm long × 70cm wide × 35cm high with several 15cm × 15cm squares. Animals were placed in the centre of the squares and were monitored for 5 minutes. The parameters for locomotion and anxiety-like behavioural activities were measured and recorded (Bădescu et al., 2016; Eilam, 2003).

Neurochemical analysis

Preparation of brain homogenates

After eight weeks of treatment, all animals were euthanized by decapitation. The brains were harvested and immediately rinsed in cold phosphate-buffered saline (PBS). Then, 0.5 g of the prefrontal cortex (n=7) was dissected on the ice tray, thawed, and homogenized in 10% phosphate buffer (0.1M, pH 7.5). The homogenates were centrifuged for 10 mins at 20,000g and 4°C. The supernatants were then obtained for neurochemical analyses.

Determination of superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA), and reduced glutathione level (GSH)

Prefrontal cortex tissue homogenates were used to measure the concentration of reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA) by spectrophotometric

assay. Reduced glutathione (GSH) level was assessed using the Ellman protocol (Ellman, 1959). Superoxide dismutase (SOD) activity and catalase (CAT) were determined as reported (Aebi, 1974; Kakkar et al., 1984). Malondialdehyde (MDA) level was determined by measuring the content of thiobarbituric acid (TBA) reactive products using the method of Mkhwanazi et al. (Mkhwanazi et al., 2014).

Analysis of inflammatory biomarkers

The concentrations of tumour necrosis factor-α (TNF-α) and interleukin (IL)-1β were quantified in the prefrontal cortex homogenates using their specific ELISA kits (Elabscience Biotechnology Co., Ltd., Houston, TX, USA) according to the manufacturer's instructions.

Brain Tissue processing for microscopic study

The prefrontal cortex (n=2) was carefully removed and weighed, post-fixed in 10% neutral buffer formalin (NBF) for 1 hour and transferred to 15% sucrose in phosphate-buffered saline (PBS) until they sunk (24 hours). Afterwards, the tissue was transferred to 30% sucrose in PBS until they sunk and finally fixed in 10% NBF for histology and immunochemistry through paraffin embedding. The tissues were sectioned at 5 μm using Leica RM 2255 microtome, cleared in xylene, hydrated in decreasing alcohols, stained with Haematoxylin and Eosin (H&E) dye, and mounted with dibutyl phthalate polystyrene xylene (Djidja et al., 2017).

Immunohistochemical (IHC) analysis

The uniform random sampling of the prefrontal cortex was used for the primary antibody (anti-GFAP). The sections from the prefrontal cortex were washed in PBS (2 x10 min) at 4°C and pre-incubated in 0.1 M PBS, 5% normal goat serum with 0.4% Triton X-100, and 1% bovine serum albumin for one hour at 4°C. Then, the sections were directly incubated in the primary antibody diluted in the PBSA -Triton (PBSAT: PBS 0.01 M, PH 7.4, 0.1% of Sodium Azide and 0.3%

Triton X 100) and prepared for 72 hours at 4 °C and under agitation. After PBST washes (2 x 10min), the sections were incubated in 0.1 M PBS containing 2% normal goat serum and biotinylated rabbit anti-goat IgG (Secondary antibody) (1:2000) for 2 hours at room temperature. They were then rinsed in PBST (2 x 10min) and incubated with the avidin-biotin complex (AB; 1:2000) for 2 hours at room followed by several washes (1 x 10 min in PBST and 2 x 10 min in Tris buffer (0.05 M, PH 7.6)). The peroxidase activity detection was carried out with 3-3' diaminobenzidine (DAB, 0.025%), 0.5% Nickel ammonium sulphate in tris buffer (0.1 M, pH 7.6) with 0.03% hydrogen peroxide. The immunoreactive reaction was stopped by washing the sections once in 0.1 M Tris buffer (10 min) and twice in 0.1 M PBS (10 min). Sections were dehydrated in progressive ethanol baths, cleared in 2 successive xylene baths, mounted onto gelatine-coated slides and coverslipped with Eukitt.

Quantification of immunostained astrocytes

Immunostained astrocytes counting was conducted under an optical microscope (Olympus BH2) connected via a CCD high-performance camera (COHU) to the Scion Image stereological software (Scion Corporation, version Beta 4.0.2) equipped computer. The counting was made in 6 sections per animal along with the rostrocaudal plan for each structure. The overall immunoreactivity was calculated for each structure, and results were represented as mean ± standard error mean (SEM).

Ultrastructural brain tissue processing

The brain tissues were initially sectioned into1 mm^3 pieces and post-fixed in buffered 2.5 % glutaraldehyde for 12 hours, washed in phosphate buffer (3 × 5 minutes), and transferred in 1 % osmium tetroxide for 2 hours. Thereafter, the tissue was washed in phosphate buffer (3 × 5 minutes), dehydrated in ascending grades of acetone solutions (30%, 50%, 75%, and 100%) for 5 minutes each, and then embedded in Durcopan (Fluka). Ultrathin sections (1 µm in thickness) of PFC were cut using an ultramicrotome (Leica Ultracut R), contrasted by uranyl acetate and lead acetate, and the prepared tissue sections were examined by transmission electron microscopy (TEM).

The TEM analysis was carried out at the Microscopy and Microanalysis Unit (MMU), the University of KwaZulu-Natal, Westville, South Africa

Statistical Analysis

Data were analyzed and presented as mean ± SEM. The differences between means were compared using one-way analysis (ANOVA), followed by Tukey's multiple comparison test to determine the statistical significance between the groups. All analyses were done using GraphPad Prism 8 for Windows (GraphPad Software San Diego, CA 92108). P <0.05 was considered statistically significant.

Results

AgNPs + HAART reduces blood glucose level in diabetic rats

Blood glucose levels increased significantly in all the diabetic groups (DC, DH and DSH) one week post-STZ vs non-diabetic group (NC). Diabetic rats administered HAART (group DH) had a significant increase ($p<0.05$) blood glucose level compared with diabetic control. In contrast, rats administered AgNPs + HAART (group DSH) had a substantial decrease in blood glucose after eight weeks of treatment vs diabetic control (DC) and diabetic treated rats only (group DH). Conversely, there was no significant difference in blood glucose levels in the non-diabetic groups (Figure 2).

AgNPs + HAART improves metabolic activities and anxiety-like behaviour

Table 1 shows the effects of HAART and AgNPs + HAART on metabolic activities and anxiety-like behaviour in non-diabetic and diabetic rats. The metabolic biomarkers significantly (water intake, urine volume, food consumption and faecal pellets) increased in the diabetic control group (DC) compared to non-diabetic control (NC). The water intake and faecal pellets significantly increased in group DH (diabetic plus HAART) (72.13±1.394) compared with the diabetic control (62.25±1.88). Interestingly, both water intake and faecal pellets significantly decreased in group DSH (diabetic + AgNPs +HAART) (48.13±2.10) compared to DH (72.13±1.394).

Table 1. Metabolic activity parameters

Groups	Water intake (cm^3)	Urine Volume (cm^3)	Food intake (mg)	Faecal pellets
ND	36.388±0.53	19.75±0.25	21.69±1.23	17.38±1.22
NH	39.13±0.22	20.63±1.19	23.78±0.88	20.00±1.19
NSH	36.63±1.15	20.75±0.31	22.96±1.61	16.63±1.32
DC	62.25±1.88^{aa}	47.00±3.09^{aa}	34.63±1.99^{a}	24.38±1.10^{a}
DH	72.13±1.394^{b}	58.50±2.19	41.30±2.47	32.25±2.23^{b}
DSH	48.13±2.10^{c}	39.25±3.69	34.85±1.72	20.38±1.45^{c}

Table 1: Effect of AgNPs + HAART on metabolic activities (water intake, urine volume, food intake, and faecal pellet number) in diabetic rats. $^{a}p < 0.05$, $^{aa}p < 0.0001$ vs NC, $^{b}p < 0.05$ v DC, $^{c}p < 0.05$ v DH. NC=nondiabetic control, NH=non-diabetic +HAART, NSH=non-diabetic+ silver nanoparticles + HAART, DC= diabetic control, DH=diabetic + HAART, DSH= diabetic +silver nanoparticles+ HAART, (n=7).

The AgNPs + HAART mitigates anxiety-like behaviours in the open field test

Figure 3 shows the effects of HAART and AgNPs + HAART on anxiety-like behaviours in non-diabetic and diabetic rats. There was a significant reduction ($p < 0.05$) in latency to leave the centre and centre square entries of group DC compared to group NC. Group DH (diabetic +HAART) had significantly reduced

latency and centre square entries compared to group DC. Notably, group DSH showed a significant ($p < 0.05$) increase (9.375±0.596) in latency compared to group DH (6.625±0.3750). The centre square entries were significantly higher in group DSH (AgNPs + HAART) (5.500± 0267) compared with group DH (3.813±0.230).

AgNPs + HAART increases locomotion activities

Figure 4 shows the effects of HAART and AgNPs + HAART on locomotion activities in non-diabetic and diabetic rats. All indices of locomotion activities significantly ($p < 0.05$) reduced in the DC group compared to group NC. The diabetic rats administered HAART (group DH) showed a significant ($p < 0.05$) reduction in locomotion compared to group DC and group DSH.

Interestingly, there was a significant increase in centre line cross in rats administered with AgNPs + HAART (group DSH) compared with group DH.

AgNPs + HAART reduces prefrontal cortex inflammatory biomarkers (TNF-α and IL-1β)

Figure 5 (a-b) shows the effects of HAART and AgNPs + HAART on inflammatory markers in non-diabetic and diabetic rats. The concentration of inflammatory biomarkers (TNF-α and IL-1β) significantly ($p < 0.05$) increased in the DC group compared to the NC group. The diabetic rat administered HAART (group DH) showed a significant ($p < 0.05$) increase in both TNF-α and IL-1β compared to the DC rat. However, the diabetic rat administered AgNPs + HAART (group DSH) showed a reduction in inflammatory biomarkers compared with HAART only but not significant.

AgNPs + HAART enhances antioxidant enzymes activities

Figure 6 (a-d) shows the effect of HAART and AgNPs + HAART on oxidative stress biomarkers. The diabetic control group had a significant ($p < 0.05$) increase in MDA level and a significant ($p < 0.05$) decrease in catalase, SOD and GSH compared with the non-diabetic control group.

Administration of HAART to the diabetic rat (DH) significantly increased MDA with a reduction in SOD and GSH compared to diabetic control. However, administration of AgNPs + HAART to diabetic animals (DSH) caused a significant decrease in MDA level and increased GSH and SOD levels compared with group DH ($p < 0.05$).

AgNPs + HAART protects GFAP-positive astrocytes in the prefrontal cortex

Figure 7A and 7B show the effect of HAART and AgNPs + HAART on GFAP-positive astrocytes. There was a significant reduction in GFAP positive astrocytes in the prefrontal cortex of diabetic control (DC) compared to non-diabetic control (NC). Administration of HAART to diabetic rats (DH) caused a significant reduction in GFAP-positive astrocytes compared with group DC. However, AgNPs + HAART administration to diabetic rats (DSH) significantly increased GFAP-positive astrocytes compared to group DH.

AgNPs + HAART prevents prefrontal cortex neuronal injury

The effect of HAART and AgNPs + HAART on prefrontal cortex neuronal cells are shown in Figure 8. The diabetic control group (DC) showed shrinkage of cytoplasm and hypertrophy of neuronal cells compared with normal control (NC). The non-diabetic groups (NH and NSH) administered with HAART and AgNPs+ HAART showed shrinkage of cytoplasm. Notably, administration of AgNPs+ HAART to diabetic rats (group DSH) showed more normal neuronal cells with few neuronal hypertrophies compared with diabetic rats administered with HAART only (group DH).

AgNPs + HAART protects ultrastructural organelles of the prefrontal cortex

Figure 9 shows the effect of AgNPs + HAART on prefrontal cortex neuronal organelles. The control group (NC) showed a normal nucleus (N) with a double-layered nuclear membrane and the presence of nucleoli. The mitochondria in the control group showed intact membrane and the presence of cristae within the mitochondria. All treated groups presented with various nucleus and mitochondrial alterations. The diabetic group (DC) and the diabetic group treated with HAART showed ruptured and vacuolated mitochondria (M) with degenerated nucleoli. However, the diabetic rats (group DSH) treated with AgNPs+ HAART showed an improved double-layered membrane, presence of nucleoli and mitochondrial cristae compared with diabetic rats treated with HAART only.

Discussion

This study examined the effect of HAART-silver nanoparticles conjugate on metabolic, behavioural, molecular, histological and ultrastructural changes associated with prolonged administration of HAART in the prefrontal cortex (PFC) of diabetic rats.

HAART is required at higher doses for a lifetime to maintain an undetectable viral load in people living with HIV, which predisposes them to systemic toxicity and metabolic disorders such as diabetes mellitus. More so, silver nanoparticles are used for a wide range of applications in the biomedical field, such as antiviral, antioxidant and antidiabetic agents due to their unique properties and ability to reduce chronic effects of these agents (Vadlapudi & Amanchy, 2017). Therefore, we hypothesized that HAART loaded

silver nanoparticles may exert a beneficial effect compared to the adverse and neurotoxic effects of HAART while improving the neurological disorders associated with its long-term use.

In this study, the diabetic rats treated with HAART had persistently increased blood glucose levels across the weeks. This result shows that the chronic administration of HAART contributed to the hyperglycaemic effect of diabetes. The development of hyperglycaemia has been reported with the prolonged use of HAART in people living with HIV (Sharma, 2014), which has been attributed to insulin resistance, mitochondrial dysfunction, and metabolic disorders (Avari & Devendra, 2017). Conversely, a significant decrease in blood glucose was observed in rats treated with HAART-silver nanoparticles conjugate (AgNPs + HAART) compared with HAART-treated rats only. This glycaemic control may be due to the potential of silver nanoparticles to increase insulin secretion, thereby promoting glucose uptake. A similar study has shown the antidiabetic activity of silver nanoparticles via up-regulation of insulin receptors and higher expression of glucokinase genes (Alkaladi et al., 2014).

The metabolic disturbances of HAART were observed as evidenced in increased defecation and water intake of diabetic rats. Literature has shown that an increase in defecation is a valuable indicator for anxiety-like behaviours and is frequently observed in highly emotional animals (Crumeyrolle-Arias et al., 2014; Hall, 1934). In this study, the administration of HAART to the diabetic rats exacerbates anxiety-like behaviour compared to diabetic control and diabetic rats treated with AgNPs + HAART. Evidence of behavioural deficits and anxiogenic effects of HAART was seen in the open field test. The centre square entries, the latency to leave the centre square, the centre line cross and the total line cross were significantly reduced in the diabetic HAART-treated group. Despite the benefits of backbones of HAART components (NRTIs and NNRTIs), they are associated with neuropsychological disturbances, fatigue, and dizziness (Romao et al., 2011). More so, chronic treatment of Efavirenz (NNRTIs) has been reported to induce an anxiety-like effect in animals and humans (Cavalcante et al., 2017; Raines et al., 2005; Romao et al., 2011).

The neurological observation in the HAART-treated rats was associated with a significant increase in the prefrontal cortex MDA level. This observation may be due to the excessive production of ROS, which occurs during the intracellular phosphorylation of NRTIs in the prefrontal cortex (Schank et al., 2021). The excessive ROS production and the reduced antioxidant enzymes CAT, SOD and GSH may promote oxidative stress and promote oxidative injury. A previous study has reported that oxidative injury promotes lipid peroxidation that compromises mitochondrial biogenesis, which has been implicated in HAART-induced mitochondrial dysfunction (Schank et al., 2021).

In this study, HAART + AgNPs alleviates the anxiety-like behaviours in the diabetic rats via improved metabolic disturbances and anxiogenic parameters in the open field, which correlated with reduced MDA and improvement in GSH, CAT and SOD. This suggests that silver nanoparticles may alleviate the anxiogenic effects of long-term administration of HAART via their antioxidant properties by reducing ROS production during the intracellular phosphorylation of HAART. This result agrees with previous findings that reported a significant antioxidant activity of silver nanoparticles (Keshari et al., 2020). In addition, an

increase in antioxidant activity has been demonstrated to improve brain cell oxidative injury and cognitive functions (Franzoni et al., 2021).

Increased oxidative stress triggers the release of pro-inflammatory cytokines (TNF-α and IL-1β), as seen in the PFC of HAART-treated and diabetic control rats. The brain tissues are particularly susceptible to oxidative stress and neuronal damage due to their low antioxidant defence system, high amount of unsaturated fatty acid, and high oxygen consumption (Salim, 2017). In the recent study, an increase in the concentration of pro-inflammatory cytokines (TNF-α and IL-1β), inhibition of antioxidants enzymes (CAT, SOD) and GSH, were associated with anxiety-like behaviour in diabetic rats treated with HAART. A similar report has established an increase in TNF-α and IL-1β were associated with mood and anxiety disorders (Quagliato & Nardi, 2018).

Furthermore, literature has shown that HIV patients receiving HAART are susceptible to type 2 diabetes mellitus and its neuropathic complications due to increased ROS production that promotes cellular toxicity and neuroinflammation (Nsonwu-Anyanwu et al., 2017; Pang et al., 2020; Sharma, 2014).

Conversely, there was a slight decrease in the PFC concentration of pro-inflammatory cytokines (TNF-α and IL-1β) in AgNPs + HAART treated rats compared with HAART treated only (although not significant). This result suggests that silver nanoparticles may mitigate the neurotoxic effect of long-term use of HAART due to their anti-inflammatory properties. This is in line with previous studies showing the anti-inflammatory and neuroprotective effects of silver nanoparticles (Seethalakshmi 2015; Tyavambiza et al., 2021).

Several studies have reported the role of PFC and astrocytic cells in anxiety disorders (Hare & Duman, 2020; Sofroniew & Vinters, 2010; Tovote et al., 2015). HAART caused astrocyte dysfunction and a decrease in PFC GFAP-positive astrocyte number in the diabetic rats. The dysfunction and decrease in astrocytes may be attributed to the neurological deficits observed in diabetic rats treated with HAART.

In this study, an increase in the PFC GFAP-positive cells was observed in AgNPs + HAART treated animals. Silver nanoparticles offer an advantage for delivering therapeutic agents due to their unique physicochemical characteristics, antioxidant, and anti-inflammatory properties (Vadlapudi & Amanchy, 2017). The improvement observed in PFC GFAP-positive cells may be attributed to the antioxidant and anti-inflammatory properties of silver nanoparticles to delay or prevent the loss of astrocytes in the PFC (Burdusel et al., 2018).

Furthermore, the administration of AgNPs + HAART to diabetic rats protects neuronal cells against oxidative injury caused by HAART and diabetes. The study has shown the therapeutic potential of silver nanoparticles in tissue restoration and regeneration (Burdusel et al., 2018).

The mechanism by which HAART exerts its neurotoxic effects has been linked with mitochondrial damage and neuronal injury (Gnanasekaran, 2020). The evidence was seen in non-diabetic and diabetic rats treated with HAART that presented with ruptured, vacuolated mitochondria and degenerated nucleoli.

However, AgNPs + HAART alleviates the anxiety-like behaviour by protecting the neuronal ultrastructural organelles (nucleus and mitochondria) in the PFC via its intrinsic anti-inflammatory and tissue restoration properties (Burdusel et al., 2018).

While the literature has reported the neurotoxic effects of silver nanoparticles (Greish et al., 2019b; Wesierska et al., 2018), there is substantial evidence that the neurotoxic effects of silver nanoparticles depend on various factors, particularly the synthesis method (Alkaladi et al., 2014; Seethalakshmi 2015). The reduction of Ag^{+} to Ag^{0} using a 1.5M trisodium citrate (TSC) concentration as a reducing and stabilizing agent with a nanoparticle size between 19-35 nm and spherical morphology may be an essential factor determining their neurotoxic effect on the PFC (Lara et al., 2010). The previous investigation of silver nanoparticles synthesis where the silver ion has been reduced to a ground state from Ag^{+} to Ag^{0}, and synthesized nanoparticles were within the small-medium nano-sized particle (20-50 nm) reported non-toxic of silver nanoparticles (Iravani et al., 2014; Lara et al., 2010; Van Dong et al., 2012). Furthermore, a study has reported that the cytotoxic effect observed in the use of silver nanoparticles is due to silver ions exposure (Wang et al., 2014). Therefore, the reduction of Ag^{+} to Ag^{0} using a 1.5M trisodium citrate (TSC) concentration as a reducing and stabilizing agent with a nanoparticle size between 19-35 nm and spherical morphology may be an essential factor determining that reduced neurotoxic effects and improved antioxidant function of nanoparticles in our research.

Conclusion

In this study, the administration of HAART aggravates anxiety-like behaviours and promotes neurotoxic effects on the PFC of diabetic rats. However, HAART conjugated with silver nanoparticles mitigates the anxiogenic effects of HAART, preserves PFC GFAP-positive cells and ultrastructural neuronal organelles, and reduces neuronal damage by reducing oxidative injury and inflammatory damage. The conjugation of silver nanoparticles and HAART as a treatment regimen in HIV is encouraged to enhance drug delivery while reducing the risk of neurological disorders (e.g., anxiety) associated with prolonged use of HAART.

Declarations

Consent for publication

All authors have read and accepted responsibility for the content of the manuscript.

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Conflict of interest disclosure:

The authors have no conflict of interest to declare.

Author's Contribution

SKL: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing-original draft, and Funding acquisition. **SOO:** Conceptualization, Methodology, and Investigation. **AD:** Methodology, Validation, Investigation, Formal analysis, and Writing- original draft. **OSF:** Formal analysis, Validation, Visualization and Writing- Review &Editing. **ESN:** Resources and Visualization, Writing- Review &Editing and supervision. **COR:** Resources, Supervision, Project administration, Writing- Review &Editing and Funding acquisition **OOA:** Conceptualization, Writing- Review and editing

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Figures

Figure 1

Illustrate experimental design. Group 1-3, designated as NC, NH and NSH were non-diabetic animals, but treated with vehicle (distilled water, 0.5 ml/100g, p.o), HAART (98.2 mg/kg, p.o), and AgNPs + HAART (24.5 mg/kg, i.p). Group 4-6, designated as DC, DH and DSH were diabetic animals treated with (distilled water, 0.5 ml/100g, p. o), HAART (98.2 mg/kg, p. o), and AgNPs + HAART (24.5 mg/kg, i.p). All rats were treated daily except for i.p groups, which were treated for 5 days per week for eight weeks. NC= non-diabetic control, NH= non-diabetic + HAART, NSH= non-diabetic + AgNPs + HAART, DC= diabetic Control,

DH= diabetic + HAART, DSH= diabetic + AgNPs+ HAART, i. p= intraperitoneal injection. p.o = per os, BLG WK= blood glucose weekly measurement, STZ= streptozotocin.

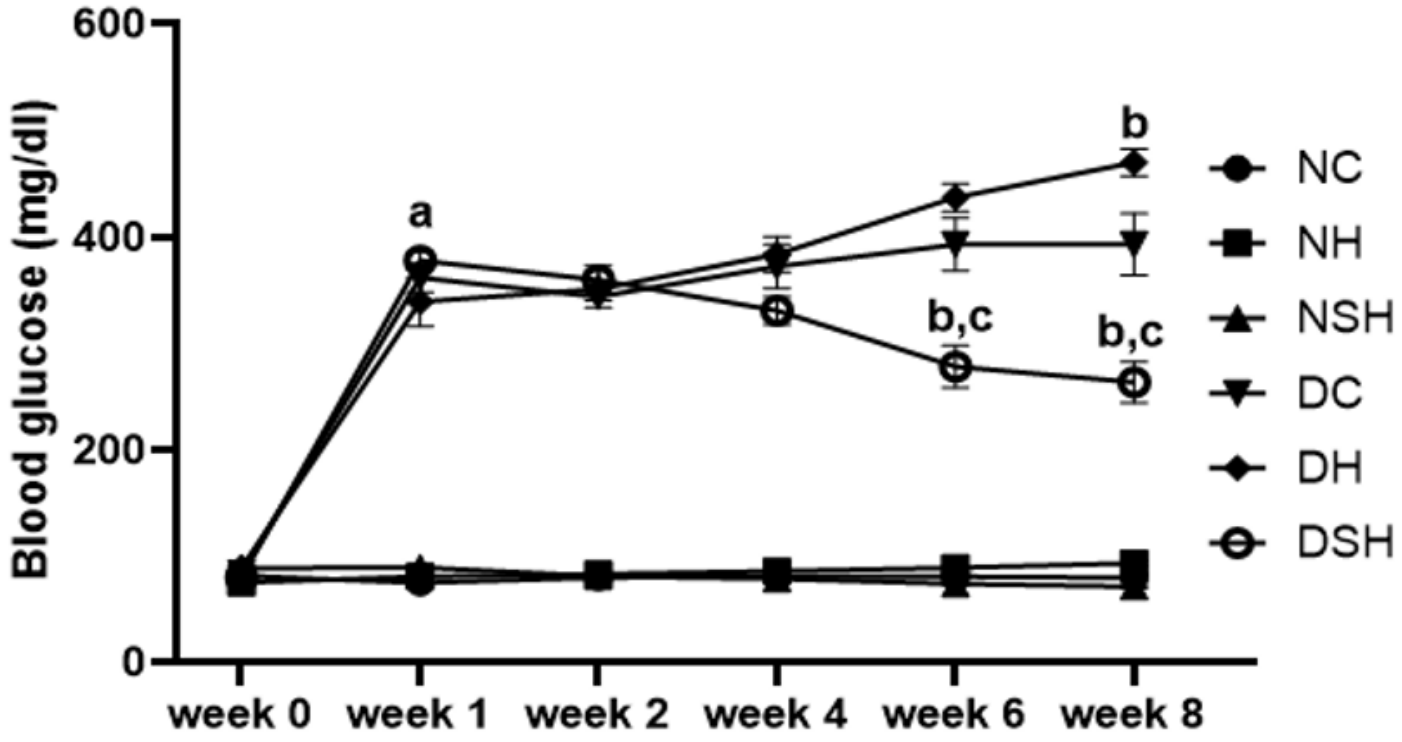

Figure 2

Illustrate the weekly changes in blood glucose level in non-diabetic and diabetic rats treated with either vehicle, HAART, or AgNPs+ HAART. NC= nondiabetic control, NH=non-diabetic + HAART, NSH=non-diabetic+ silver nanoparticles + HAART, DC= diabetic control, DH=diabetic + HAART, DSH= diabetic +silver nanoparticles+ HAART. [a] vs NC; [b]$p < 0.05$ v DC, [c]$p < 0.05$ v DH, (n=7).

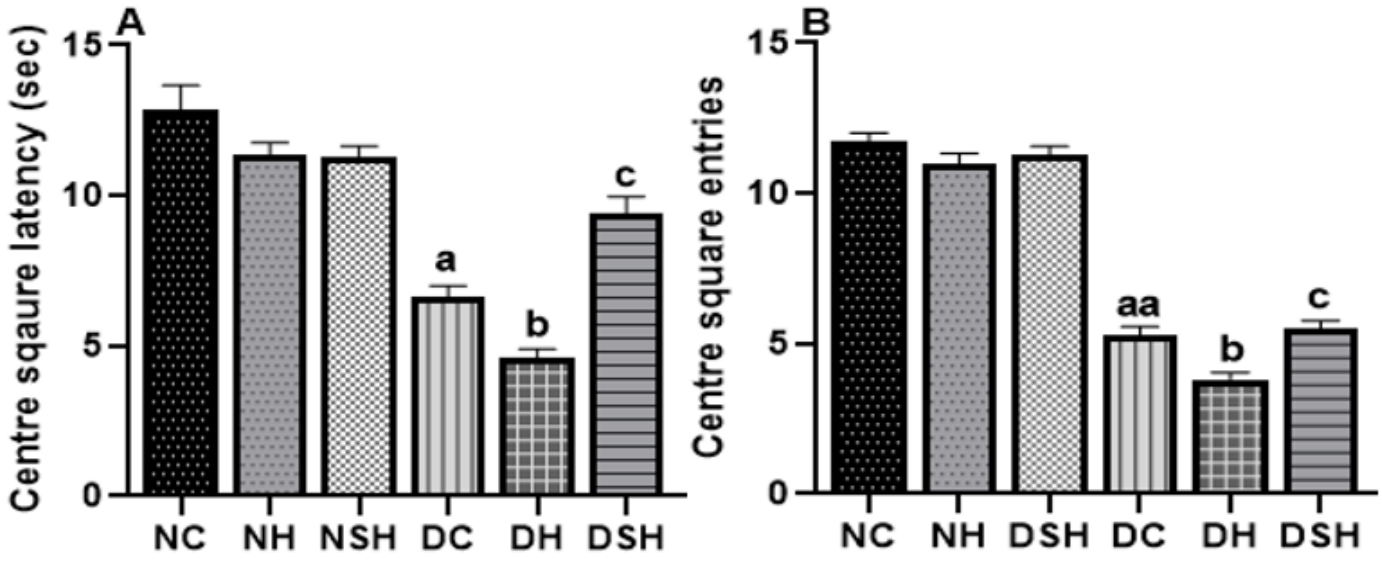

Figure 3

Effect of AgNPs + HAART on anxiety-like behaviour in diabetic rats. $^{a}p < 0.05$, $^{aa}p < 0.0001$ vs vs NC, $^{b}p < 0.05$ v DC, $^{c}p < 0.05$ v DH. NC= nondiabetic control, NH=non-diabetic + HAART, NSH=non-diabetic+ silver nanoparticles + HAART, DC= diabetic control, DH=diabetic + HAART, DSH= diabetic + silver nanoparticles+ HAART. A= center square latency, B= center square entries, (n=7).

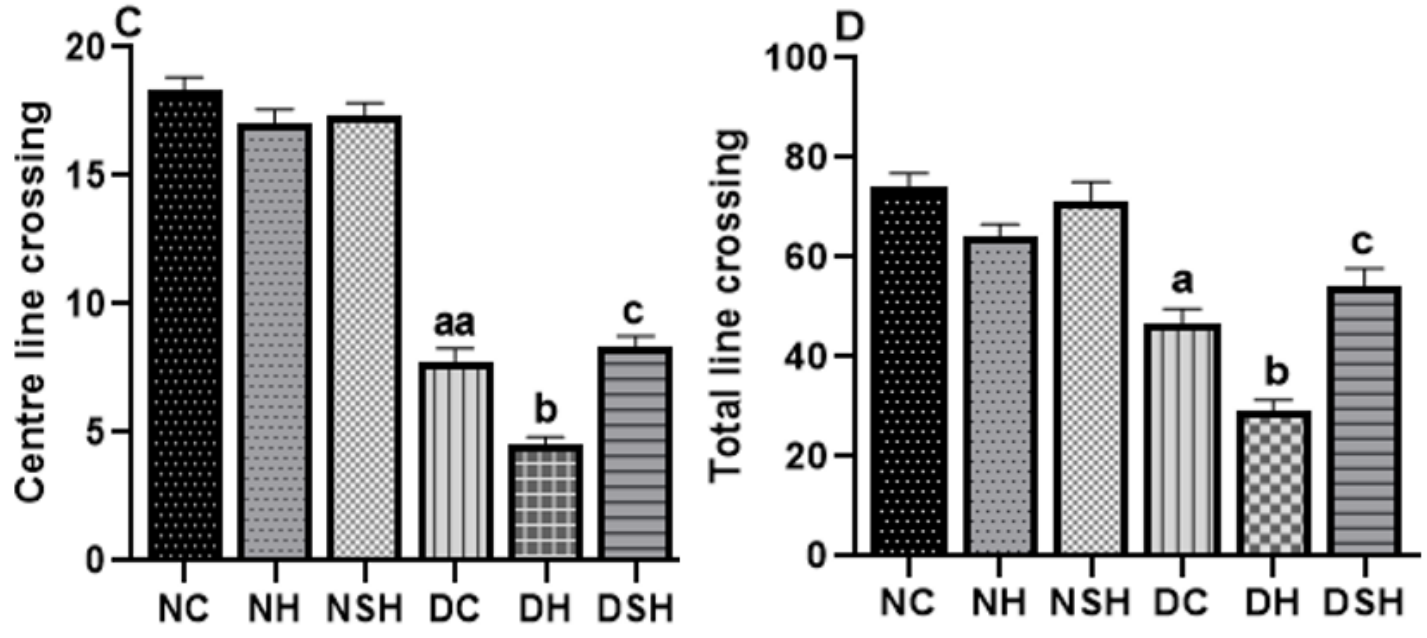

Figure 4

Effect of AgNPs + HAART on locomotion in diabetic rats. [a]p < 0.05, [aa]p < 0.0001 vs NC, [b]p < 0.05 v DC, [c]p < 0.05 v DH. NC= nondiabetic control, NH=non-diabetic + HAART, NSH=non-diabetic+ silver nanoparticles + HAART, DC= diabetic control, DH=diabetic + HAART, DSH= diabetic +silver nanoparticles+ HAART. A= Centre line crossing, B= total line cross, (n=7).

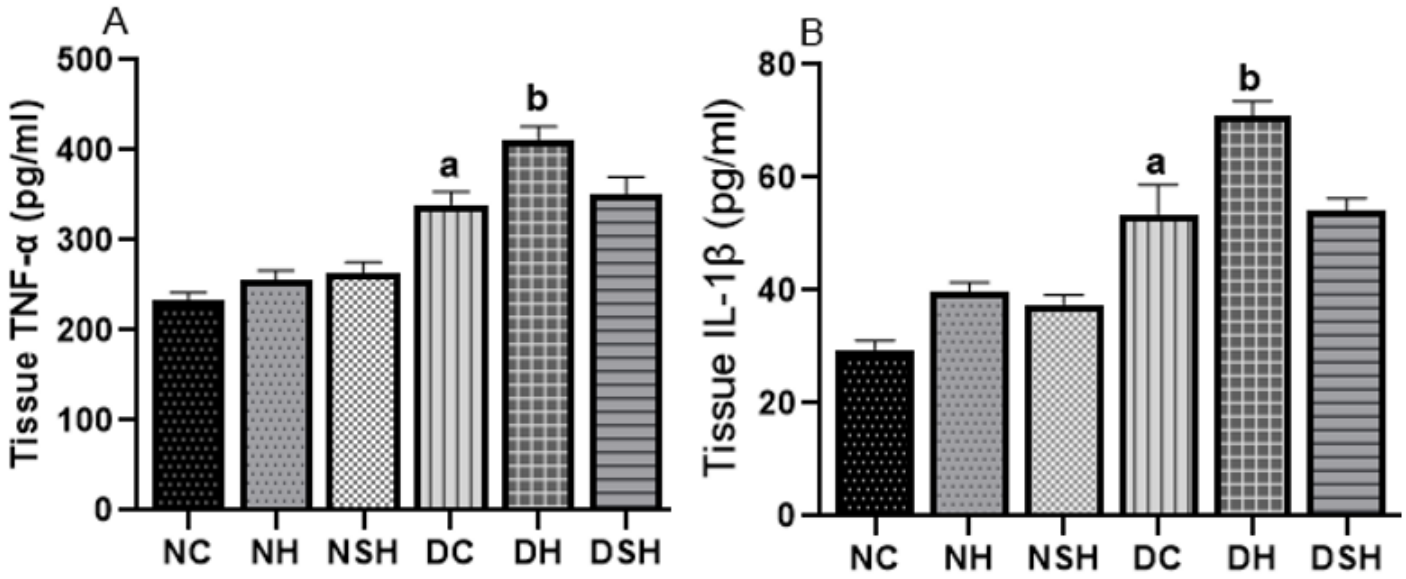

Figure 5

Effect of AgNPs + HAART on inflammatory response in the Prefrontal cortex of diabetic rats. [a]p < 0.05 vs NC, [b]p < 0.05 v DC. NC= nondiabetic control, NH=non-diabetic + HAART, NSH=non-diabetic+ silver nanoparticles + HAART, DC= diabetic control, DH=diabetic + HAART, DSH= diabetic +silver nanoparticles+ HAART. TNF-α = tumour necrosis factor- alpha, IL-1β = interleukin-1 beta, A= TNF-α, B= IL-1β, (n=7).

Figure 6

Effect of HAART on oxidative stress in the Prefrontal cortex of diabetic rats. [a]p < 0.05 vs NC, [b]p < 0.05 v DC, [c]p < 0.05 v DH. NC= nondiabetic control, NH=non-diabetic + HAART, NSH=non-diabetic+ silver nanoparticles + HAART, DC= diabetic control, DH=diabetic + HAART, DSH= diabetic +silver nanoparticles+ HAART. A= Catalase, B= Malondialdehyde, C=superoxide dismutase (SOD), D= reduced glutathione (GSH), (n=7).

Figure 7

A: Prefrontal cortex GFAP-positive astrocytes in diabetic and nondiabetic groups.

NC= nondiabetic control, NH=non-diabetic + HAART, NSH=non-diabetic+ silver nanoparticles + HAART, DC= diabetic control, DH=diabetic + HAART, DSH= diabetic +silver nanoparticles+ HAART. Black arrow indicates GFAP-positive astrocyte

B: Effect of AgNPs + HAART on GFAP positive astrocytes in the Prefrontal cortex. NC= nondiabetic control, NH=non-diabetic + HAART, NSH=non-diabetic+ silver nanoparticles + HAART, DC= diabetic control, DH=diabetic + HAART, DSH= diabetic +silver nanoparticles+ HAART, GFAP= glial fibrillary acidic protein. $^{a}p < 0.05$ v NC, $^{b}p < 0.05$ v DC, $^{c}p < 0.05$ v DH. Black arrow indicates immunostained astrocytes, (n=2).

Figure 8

Effect of AgNPs + HAART on Prefrontal cortex neuronal cell. NC= nondiabetic control, NH=non-diabetic + HAART, NSH=non-diabetic+ silver nanoparticles + HAART, DC= diabetic control, DH=diabetic + HAART, DSH= diabetic +silver nanoparticles+ HAART. Black arrow = normal neuronal cell, red arrow = neuronal atrophy, green arrow= neuronal hypertrophy and blue arrow= cytoplasmic shrinkage (n=2).

Figure 9

Effect of AgNPs + HAART on Prefrontal cortex neuronal organelles. NC= nondiabetic control, NH=non-diabetic + HAART, NSH=non-diabetic+ silver nanoparticles + HAART, DC= diabetic control, DH=diabetic + HAART, DSH= diabetic +silver nanoparticles+ HAART. N indicates Nucleus (short red arrow = nuclear membrane, long red arrow= nucleoli), M indicates Mitochondria (blue arrow= mitochondrial cristae) (n=2).