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Cerebellar Perturbations of Combination Antiretroviral Therapy (CART): Can Bioflavonoids Help?

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ABSTRACT

Long term usage of combination anti-retroviral therapy (cART) has been associated with neurological disorders as a result of varying toxicities. This study was therefore designed to investigate the therapeutic potential of selected flavonoids (Naringenin and Quercetin) on cART-induced cerebellar disorders. Seventy Wistar rats were divided into seven groups as control, Naringenin (50 mg/kg), Quercetin (50 mg/kg), 24 mg/kg cART (Efavirenz + Lamivudine + Tenofovir regimen), 24 mg/kg cART + 50 mg/kg Naringenin, 24 mg/kg cART + 50 mg/kg Quercetin and 1% v/v dimethyl sulphoxide (DMSO) groups. The animals were euthanized on the 57th day, processed for oxidative stress markers, and basic histology. Results showed that the Purkinje cells were very distinct in groups that received cART with Naringenin, and cART with Quercetin, whereas the animals that received cART alone showed neurodegenerative changes in the Purkinje cells. Likewise, the malondialdehyde (MDA) levels increased significantly ($p < 0.0001$) in animals that received cART alone compared to control. There was also a concomitant significant decrease in the superoxide dismutase ($p < 0.05$), and catalase ($p < 0.05$) in cART treated group compared to control. Animals that received both cART and bioflavonoids had marked increase in antioxidant enzymes and decrease in MDA levels compared to cART treated group. Results of this study demonstrate that Naringenin and Quercetin have therapeutic benefits by potentiating the activities of antioxidant enzymes which prevents the onset/deleterious impact of reactive oxygen species on the cerebellum of the Wistar rat.

Key words: *cART, Naringenin, Quercetin, Neurodegeneration, Oxidative Stress*

INTRODUCTION

Human immunodeficiency virus (HIV) was discovered in the early 1980's and since then has infected millions of persons worldwide. All HIV-infected persons are at risk of illness and death from different infections as a result of the immune suppression manifestations of acquired immune defiency syndrome (AIDS) (Maartens et al. 2014). Antiretroviral therapies are geared towards reducing HIV replication and destruction of the immune system with progression to AIDS. A variety of pharmacologic agents have been developed to treat HIV infection (Klatt 2003).

The combination antiretroviral therapy (cART) which was introduced in 1996 has drastically reduced the morbidity and mortality associated with the HIV infection (Ramana et al. 2013), by maintaining viral load below detection levels, thus preventing the onset

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of AIDS (Walensky and Paltiel 2006). There are five classes of antiretroviral therapy (ART) drugs but in most cases, cART therapy involves the concurrent use of a combination of three or more ART drugs, including nucleotide reverse transcriptase inhibitors, non-nucleotide reverse transcriptase inhibitors and protease inhibitors (Feeney 2011). Among the regimens recommended by the World Health Organization (WHO) as first-line ART are those consisting of tenofovir disoproxil fumarate (Tenofovir) and efavirenz combined with either lamivudine or emtricitabine (WHO 2016). However, it has been suggested that exposure to antiretroviral medications may have marked adverse effects, independent of HIV status and these adverse effects have been shown to compromise quality of life (Ritesh Kumar 2010). Low concentrations of antiretroviral drugs penetrate the blood brain barrier (Letendre et al. 2004), thus, the long term use becomes detrimental to the central and peripheral nervous system (Chen et al. 2011). These variegated neurological complications are grouped under the term HIV-associated neurocognitive disorders (HAND). Clinical features of HAND include cognitive deficits and also particularly cerebellum-associated motor symptoms and associated with neurodegeneration of cerebellar neurons (Brandmann et al. 2014). Most of these neurodegeneration are due to neurotoxicities exacerbated by excess reactive oxygen species (ROS) (Sharma 2014).

Nature has provided a wealth of resources that can be used to combat infections and treat diseases. Among these resources are, bioflavonoids including Quercetin reported to possess anti-HIV properties (Kurapati et al. 2016). Quercetin, a polyphenolic bioflavonoid abundantly found in vegetables, fruits, red wines, and black berries have been attested to possess potent antioxidant, anti-inflammatory and neuroprotective properties and has been shown to protect nerve cell senescence in Parkinson's disease (Jeon and Ahn 2015). Naringenin is another natural flavanone, richly found in citrus and grapefruits, and exhibits antioxidant potential, superoxide scavenging, antiapoptotic, antiatherogenic and metal chelating activities (Rani et al. 2016).

The present study investigates the potential therapeutic effects of Naringenin and Quercetin on highly active antiretroviral therapy (cART)-induced cerebellar disorders in an experimental animal model.

MATERIALS AND METHODS

Experimental Protocols

All animal procedures used in the experiments were approved by the College Health Research Ethics Committee of College of Medicine of the University of Lagos with protocol number CMUL/HREC/03/17/113. They were housed in well-ventilated plastic cages which were kept and maintained under standard laboratory conditions. They also had access to clean tap water and food *ad libitum*.

Drug Administration

cART (Tenofovir 300mg, Lamivudine 300 mg and Efavirenz 600 mg all in one pill) manufactured in India by Macleods Pharmaceuticals was obtained from the AIDS Pediatric Initiative of Nigeria (APIN) Clinic, Lagos University Teaching Hospital, Lagos, Nigeria. While, Quercetin (Cat No: Q4951-100G), Naringenin (Cat No: W530098-500G) and dimethyl sulfphoxide (DMSO) (Cat No: 317275-500mL) were purchased from Sigma-Aldrich, South Africa.

Experimental Design

Seventy adult male Wistar rats weighing 220-250 g were randomly divided into seven groups (C, DMSO, N. Q, H, HN, and HQ) of 10 animals each. Group C received 1 ml distilled water; Group DMSO received 1% v/v of DMSO, Group N received 50 mg/kg Naringenin (Chtourou et al. 2015); Group Q received 50 mg/kg Quercetin (Halder et al. 2016); Group H received 24 mg/kg cART (Tenofovir 300 mg + Lamivudine 300 mg + Efavirenz 600 mg); Group HN received 24 mg/kg cART and 50 mg/kg Naringenin; Group HQ received 24 mg/kg cART and 50 mg/kg Quercetin. Both Naringenin and Quercetin were dissolved in 1% v/v DMSO.

The administration was via oral route for 56 days. At the end of the administration, the animals were euthanized and the cerebella were isolated for further investigation of oxidative stress markers and basic histology

Tissues processing for Basic Histology

Cerebellar tissue samples were processed for paraffin embedding, sectioned at 4 µm using a Thermo Scientific HM 325 rotatory microtome (CE Bright Company Ltd. Huntington England), and stained with haematoxylin and eosin (H&E).

Tissue Processing for Biochemical Parameters

Immediately after euthanasia and removal of cerebella, sections of the tissues were stored in Eppendorf tubes. 0.5 g of each cerebellar tissue was homogenized using tissue homogenizer in 4.5 mL of 0.4M sodium phosphate buffer (pH 7.0), centrifuge at 3500 rpm for 10 min, and the supernatant removed for the estimation of biochemical parameters.

Assessments of Superoxide Dismutase, Catalase and Glutathione

Total superoxide dismutase (SOD) activity in tissue homogenates was determined following the procedure of Marklund and Marklund (1974) with some modifications. The method is based on the ability of SOD to inhibit the autoxidation of pyrogallol. In 970 µL of buffer (100mMTris-HCl, 1mM ethylene diamine tetraacetic acid (EDTA), pH 8.2), 10 µL of homogenates and 20 µL pyrogallol (13 Mm) were

mixed. Assay was performed in thermostated cuvettes at 25°C and changes of absorption were recorded by a spectrophotometer at 480 nm. One unit of SOD activity was defined as the amount of enzyme which can inhibit the auto-oxidation of 50% of the total pyrogallol in the reaction.

Catalase (CAT) was assayed calorimetrically at 620 nm and expressed as moles of hydrogen peroxide (H_2O_2) consumed/min/mg protein. The reaction mixture (1.5 mL) contained 1.0 mL of 0.01M pH 7.0 ml of TCA (10%), and 1.0mL thiobarbituric acid (0.67%). All the test tubes were placed in a boiling water bath for a period of 45 minute. The tubes were shifted to ice bath and then centrifuged at 2500×g for 10 minutes. The amount of malondialdehyde (MDA) formed in each of the samples was assessed by measuring the optical density of the supernatant at 532 nm. The results were expressed as the nmol MDA formed/gram tissue by using a molar extinction coefficient of $1.56\times10^5\ M^{-1}\ cm^{-1}$.

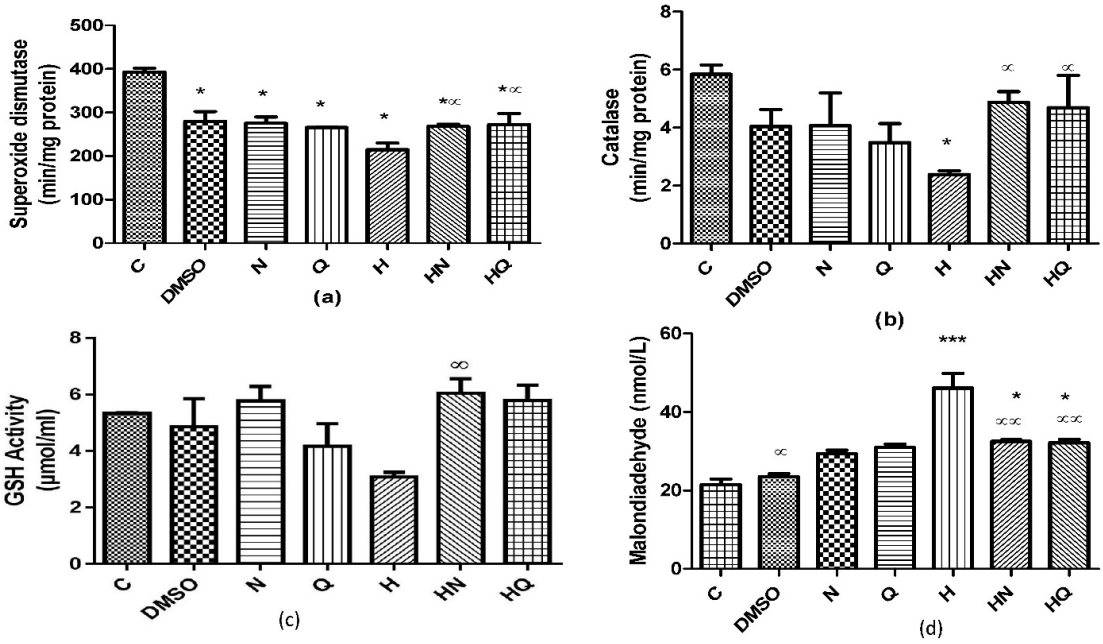

Figure 1: Oxidative stress markers: Control (C), Dimethyl sulphoxide (DMSO), Naringenin (N), Quercetin (Q), cART (H), cART+ Naringenin (HN), and cART+ Quercetin (HQ) groups. *p<0.05 = significantly different from control; ***P<0.0001 significantly different from control; άp<0.05=significantly different from cART; ά άp<0.001 significantly different from cART

phosphate buffer and 0.4 mL of 2M H_2O_2. The reaction was stopped by the addition of 2.0 mL of dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratio) (Sinha 1972).

Glutathione (GSH) was determined by the method described by Ellman (1959). To the homogenate added 10% trichloroacetic acid (TCA) (equal volume) and centrifuged. 1.0 mL of supernatant was treated with 0.5 mL of Ellman's reagent (19.8 mg of 5, 5'-dithiobisnitro benzoic acid in 100 mL of 0.1% sodium nitrate) and 3.0 mL of phosphate buffer (0.2M, pH 8.0). The absorbance was read at 412 nm.

Determination of Lipid Peroxidation through Assessment of Malondialdehyde

The assay for membrane lipid peroxidation was done by the method of Tsikas (2017) with some modifications. The reaction mixture in a total volume of 3.0 mL contained 1.0 mL tissue homogenate, 1.0

Statistical Analysis

Statistical analysis was done using the GraphPad Prism 7.0 (Graph Pad Software, La Jolla, USA) performing one-way analysis of variance and Tukey`s multiple comparison post-hoc to evaluate statistical differences, Value of $p < 0.05$ was considered significant.

RESULTS

Morphological Observations: Effect on Oxidative Stress Markers

The study evaluated oxidative stress markers including SOD, CAT, GSH and MDA analyses. There was a significant ($p < 0.05$) decrease in the SOD levels of all groups compared to control. There was also a significant ($p < 0.05$) decrease in SOD levels of cART group compared to the animals that received

both cART and Naringenin, and the cART and Quercetin (Figure 1a).
The CAT levels in animals that received cART alone was significantly ($p < 0.05$) decreased compared to control, cART and Quercetin, and the cART and Naringenin groups (Figure 1b). There was also a significant ($p < 0.05$) increase in GSH levels of animals that received both cART and Naringenin compared to those that received cART alone (Figure 1c).
MDA levels also increased significantly ($p < 0.0001$) in animals that received cART only compared to control. Likewise, animals that received a concomitant administration of cART with Naringenin, and cART with Quercetin, significantly ($p < 0.05$) increased MDA levels compared to control (Figure 1d).

Histomorphological Changes after Treatment
The morphology and cyto-architecture of the neuronal cells of the cerebellar tissues of the Wistar rats in the control, DMSO, Naringenin and Quercetin groups were intact. But the animals that received cART alone showed pale stains in the Purkinje cell which represents later form of cell death. Whereas, the Purkinje cells were protected in the groups that received cART with Naringenin (HN), and cART with Quercetin (HQ) (Figure 2). It was also observed that the granular cells in groups that received Quercetin, a combination of cART and Naringenin (HN), and cART and Quercetin (HQ) were sparsely distributed compared to other groups.

DISCUSSION

In this study, it was observed that the use of cART in Wistar rats led to neuronal loss of cerebellar Purkinje cells. This damage may be due to the myriad of toxicities caused by cART which can be linked to the cellular stresses such as oxidative stress, as well as the drug-drug reactions of the multiple drugs (TFV/3TC/EFV) that make up the cART regimen (Diop et al. 2013). Though, neurons are not directly affected by this toxicity, direct cART-toxicity affect the resident cells of the central nervous system, macrophages and microglia, leading to the prolonged activation of these cells that further result into neuronal damage (Minghetti 2005; Appel et al. 2015). Our findings are in tandem with reports on the degenerative effects of oxidants on cerebellar Purkinje neurons (Chen et al. 2003). It is plausible that free radicals are messengers signalling such neuronal apoptosis via an oxidative stress mechanism. Oxidative stress occurs as a result of malfunctioning mitochondria in the cytosol but eventually affects the nuclear integrity causing deoxyribonucleic acid (DNA) fragmentations (Henkel and Solomon 2018). Hence, apoptosis is induced to prevent already stressed cells from proliferating and running amok (Kannan and Jain 2000). However, hyper-induction of apoptosis will lead to neurodegeneration (Lauritzen et al. 2010) as seen in Parkinson`s disease and Alzheimer`s disease (Amor et al. 2010).
The increased MDA levels as observed in this study is synonymous to increased lipid peroxidation

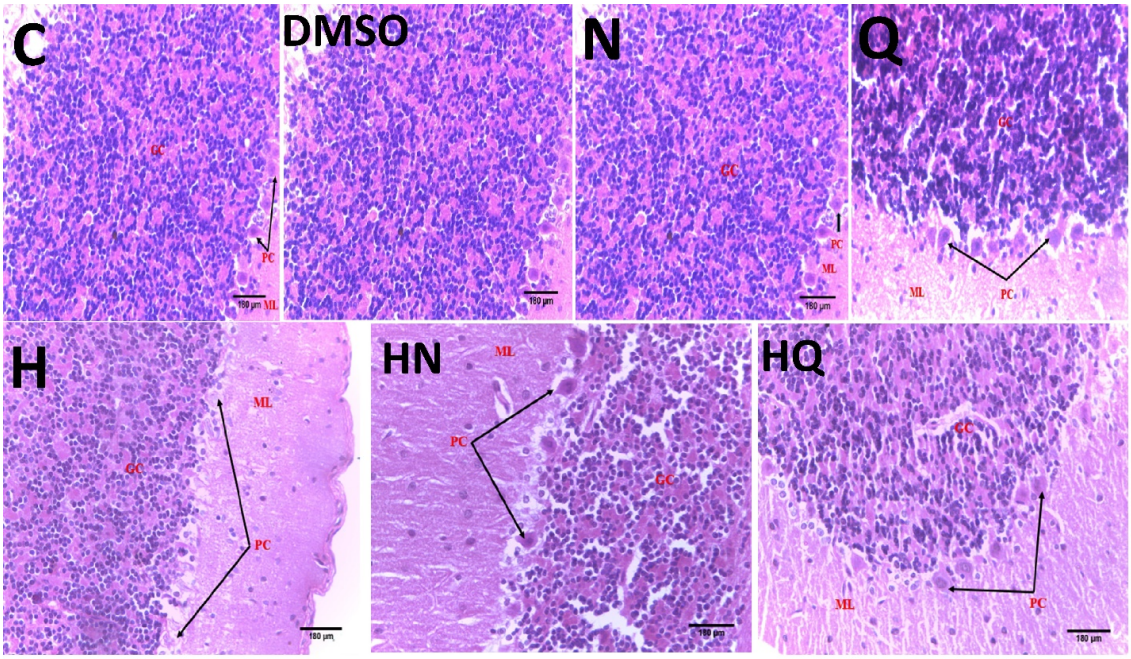

Figure 2: Histomorphology of the cerebellum showing cerebella profile of neuronal cells; ML Molecular layer, PC Purkinje cells, GC Granule cells stained with H an E and captured at x400.

(Esterbauer et al. 1991). One of the major target sites for MDAs are the proteins of the mitochondrial electron transport chain (ETC), which includes succinic acid dehydrogenase (Aitken et al. 2012). When these lipid aldehydes bind to these free proteins, it interferes with the regulated ETC, leading to the formation of superoxide anion which will dismutate to hydrogen peroxide inducing the production of yet more electrophilic lipid aldehydes that again target the ETC and so the cycle continues (Aitken et al. 2013). Thereby altering the morphology of the neuronal membranes, as well as disrupting metabolism leading to impaired synapses and ultimately - cell death (Tabe et al. 2015). Moreover, the decreased antioxidant enzymes in the cerebellum of animals treated with cART is an anecdotal evidence of an imbalance in the redox equilibrium resulting in oxidative stress (Colado Simao et al. 2015).

It is a well-established fact that oxidative stress mechanism makes the neurons more vulnerable to degeneration and to the development of neurodegenerative disorders (Cui et al. 2012; Sharma 2014). Thus, neuronal cells, as common with all cell types have developed an elaborate antioxidant defense system consisting of enzymes such as CAT, SOD, and reduced GSH that scavenge and suppress the formation of reactive oxygen species (Wilcox 2004). The findings from this study, suggest clearly the neuroprotective ability of Naringenin, and Quercetin is attributable to its potentiating effects on endogenous antioxidant enzymes which successfully prevents the onset/impact of ROS (Nishimura et al. 2013). This is evidenced by the normalcy of the cyto-architecture of the Purkinje cells in the cerebellum of animals co-administered cART with Naringenin, and Quercetin. The above findings is in agreement with the findings of Rivera et al. (2008) and Raza et al. (2013), who submitted that Quercetin and Naringenin play significant protective role against neuronal brain injury.

CONCLUSION

The inevitable use of highly active antiretroviral therapy (cART) in the treatment of HIV infection leads to the production of excessive ROS causing cerebellar neuronal loss and ultimately, neurodegeneration. However, the use of bioflavonoids such as Quercetin and Naringenin mitigates the deleterious effects of cART by restoring an equilibrium in redox activity.

Conflict of Interest
None declared.

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