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Oxidative stress induced by chronic administration of Efavirenz on the intracranial visual relay centers of adult Wistar rats

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Abstract

The biochemical effects of chronic administration of efavirenz commonly used as part of highly active antiretroviral therapy (HAART) for the treatment of Human Immunodeficiency Virus (HIV) type-1 on the intracranial visual relay centres (namely superior colliculus and lateral geniculate body) of adult Wistar rats were carefully studied. The rats of both sexes (n=30), with an average weight of 200g were randomly assigned into treatment (n=15) and control (n=15) groups. The rats in the treatment group received the recommended dose of 600mg/70kg body weight of efavirenz dissolved in distilled water daily for 30 days (thirty days) through the orogastric tube. The control group received equal volume of distilled water daily for 30 days through the same route. The rats were fed with grower's mash obtained from Edo Feeds and Flour Mill Limited, Ewu, Edo state, Nigeria and given water liberally. The rats were sacrificed by cervical dislocation method on the thirty-first day of the experiment and the superior colliculus and lateral geniculate body were carefully dissected out, weighed using the Mettler Toledo weighing balance and homogenized. The homogenates were centrifuged at 3500rpm for 5 minutes and the clear supernatants were collected using a micropipette which was then transferred into an empty specimen container and refrigerated till needed for the biochemical assays. The findings indicated that the Malonyldialdehyde (MDA) and Superoxide Dismutase (SOD) were consistent. MDA as a non-enzyme biomarker of oxidative stress is higher on the treated tissues, with statistically significant ($P<0.05$) difference observed. SOD is consistently lower in the treated tissues with statistical significant ($P<0.05$) difference as compared with the control group in this experiment. Chronic administration of efavirenz may therefore have an adverse effect on the visual sensibilities by affecting the superior colliculus and lateral geniculate body of adult Wistar rats.

Keywords: Biochemical effects; efavirenz; superior colliculus; lateral geniculate body; Wistar rats.

Introduction

Efavirenz is an antiretroviral drug that belongs to the class of drugs called non-nucleoside reverse transcriptase inhibitor (NNRTI) used as part of highly active antiretroviral therapy (HAART) for the treatment of human immunodeficiency virus (HIV) type-1 (AHFS, 2007). Efavirenz has been found to be effective in many combination regimes for the treatment of HIV infection, both in previously untreated and in treated individuals. It has been combined successfully with nucleoside consisting of lamivudine or emtricitabine plus abacavir, didanosine, stavidine, tenofovir or zidovudine to achieve virologic suppression in a high percentage of recipients (Staszewski *et al.*, 1999; Gulick *et al.*, 2006). Most antiviral agents do not efficiently penetrate the blood brain barrier (BBB) or are actively transported out of the central nervous system (Schranger and D'Souza, 1998). Even after antiviral treatment that successfully controls virus in the treatment compartments, the central nervous system may suffer continuing damage induced by HIV infection (Fox *et al.*, 2000). Efavirenz may be taken once a day without regards to meal and it can penetrate the central nervous system and spinal fluids (AIDS INFONET, 2007; Puzantian, 2002).

Some adverse effect in the central nervous system has been commonly associated with efavirenz (Ruiz *et al.*, 1999). The most common central nervous system effects include confusion, insomnia, abnormal vivid dreams, dizziness and headache. Efavirenz has emerged as cornerstone of highly active antiretroviral therapy (HAART) regimens. The side effect profile of the drug is generally regarded as satisfactory. However, there are conflicting study results in the medical literature as well as conflicting studies from patients and

physicians regarding the neuropsychiatric problems associated with efavirenz (Baker, 2006). Lipodystrophy, moderate or severe pain, abnormal vision, arthralgia. asthenia, dyspnea, gynecomastia, myalgia, myopathy and tinnitus have been reported concerning efavirenz (AHFS, 2007).

The superior colliculus and lateral geniculate body constitutes the intracranial visual relay centres. The lateral geniculate body in mammals is considered as part of the thalamic nuclei for processing visual information (Altman and Bayer, 1981). In rats, the lateral geniculate body receives input from the geniculate leaflet, which participates in the regulation of circadian function through its projection to the circadian pacemaker of the hypothalamus (Moore and Card, 1984). The superior colliculus is concerned with ocular movement. Such movements can result from stimulation of a wide area in the pretectal and tegmental regions of the brain. The superior colliculus controls and regulates many movements of the eye and head. It acts as an integrative center subserving visual perception. Thus, it also has a role in certain aspects of vision. Its major role is to co-ordinate responses evoked by a variety of sensory signals with behavioural movements that directs the head, eyes and ear towards the environmental stimulus. Thus, the superior colliculus has a critical role in visual localization, orientation tracking movements, accommodation and papillary reflex. Its superficial layers are concerned with vision (Reczkowski and Diamond, 1978), and its deep layer has been implicated in eye movements and somesthetic input (Altman and Bayer, 1981).

It has been observed in monkey that the neurons in the superior colliculus are involved in a somatosensory motor feedback loop that monitors the force of the active muscles together with the spatial position of the limb that is required for proper interaction with an object (Nagy *et al.*, 2006). Multisensory depression is a fundamental index of multisensory integration in the neurons of the superior colliculus. Nitregic interneurons play a role in refining the cortico-collicular projection patterns that are believed to be essential for superior colliculus output neurons. It is engage in multisensory integration and to support normal orientation responses to cross modal stimuli (Stein *et al.*, 2009). The loss of these cortical influences permits visual orientation behaviour in the presence of a normal disruptive auditory stimulus (Jiang and Stein, 2003).

The superior colliculus neurons play some spatial- temporal filter properties that are closely similar to those of their retina as well as those of their inputs from the cortical visual motion detector areas, suggesting their common role in motion analysis and related behavioural actions (Waleszczyk *et al.*, 2007).

Cortical structures such as the medial and lateral geniculate bodies, inferior and superior colliculi have higher glucose utilization than other structures (Siesjo, 1978). There is a correlation between functional activity and metabolic rate such as in the visual and auditory system (Siesjo, 1978). Since efavirenz crosses the blood-brain barrier, it is relevant to investigate its biochemical effect on the superior colliculi and lateral geniculate body. It is probable that the adverse effects of efavirenz on dizziness and headache may be due to direct effect of efavirenz on the superior colliculus and lateral geniculate body. This present study was to elucidate the biochemical effects of chronic administration of efavirenz on the intracranial visual relay centre of adult Wistar rats.

Materials and Methods

Animals: Thirty adult Wistar rats of both sexes with average weight of 200g were randomly assigned into two groups; control (n=15) and treatment (n=15). The rats were obtained and maintained in the Animal Holding of the Department of Anatomy, School of Basic Medical Sciences, University of Benin, Benin City, Edo State, Nigeria. They were fed with grower's mash obtained from Edo Feeds and Flour Mill Limited, Ewu, Edo State, Nigeria and given water liberally. Efavirenz was obtained from the President Emergency Plan for AIDS Relief (PEPFAR) Unit, University of Benin Teaching Hospital, Benin City, Edo State, Nigeria.

Drug Administration: The rats in the treatment group received the recommended dosage of 600mg/70kg body weight of efavirenz dissolved in distilled water for thirty days through orogastric tube administration while the control rats received equal volume of distilled water through the same route and for the same period. The body weights of both groups were measured using Mettler Toledo weighing balance before and during the period of treatment.

Dissection of the brain, superior colliculus and lateral geniculate body: The rats in both groups were sacrificed by cervical dislocation and the skull was quickly opened with the aid of a pair of bone forceps to expose the brain. The superior colliculi and lateral geniculate bodies were carefully dissected out, weighed using the Mettler Toledo weighing balance and the biochemical techniques of the various assays were carried out.

Preparation of sample: The different samples (superior colliculus and lateral geniculate body) from the experimental animals were dissected out, homogenized in a mortar and pestle with a pinch of acid washed sand and a total of 5mls of normal saline (0.9%) added sequentially during the homogenization process. The homogenates were centrifuged at 3500rpm for 5 minutes with the aid of a centrifuge. The clear supernatants were collected using a micropipette and transferred into an empty specimen container and refrigerated till needed for the assays.

Superoxide dismutase (SOD) assay: The SOD activities in these tissues were determined by the method of Misra and Fridovich (1972). An aliquot (0.4ml) of the supernatant was added to 5ml of 0.05M carbonate buffer (pH 10.2) equilibrated in the spectrophotometer for 2–3 minutes. The reaction was then initiated by the addition of 0.6ml of freshly prepared 0.3mM adrenaline as substrate to the buffered-supernatant mixture which was quickly mixed by inversion and the absorbance taken. The reference corvette contained 5ml of the carbonate buffer, 0.6ml of the substrate and 0.4ml of distilled water. The increase in absorbance at 420nm due to the adenochrome formed was monitored every 30 seconds for l20 seconds. 1 unit of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the auto-oxidation of adrenaline to adenochrome during 120 seconds.

Protocol:

Tubes	Blank	Test	Reference
Distilled H_2O	3.0ml	-	0.2ml
Sample	-	0.2ml	-
Carbonate buffer	-	2.5ml	2.5ml
Adrenaline	-	0.3ml	0.3ml
Solution			

It was allowed to equilibrate before adding adrenaline solution and read immediately after addition of 0.3ml of adrenaline. Take absorbance at 420nm.

Catalase assay: The method of Cohen *et al.* (1970) was adopted. Aliquots of the homogenate supernatant (0.5ml) were added into ice cold test tubes while the blank contained 0.5ml distilled water. The reaction was initiated by adding sequentially, at fixed interval, 5ml of cold 30mM hydrogen peroxide and was mixed thoroughly by inversion. The test samples and the blank were taken one at a time, and 7ml of 0.01M potassium permanganate was added which was mixed twice by inversion and absorbance at 480nm. It was read within 30-60 seconds. The spectrophotometer standard was prepared by adding 7ml of 0.01M potassium permanganate to a mixture of 5.5ml of 0.05M phosphate buffer with pH 7.0 and 1ml of 6M-tetraoxosulphate VI acid solution. The spectrophotometer was zeroed with distilled water and the activity of the enzyme was estimated.

Protocol:

Tubes	Test	Blank
Sample aliquots	0.5ml	-
Distilled H_2O	-	0.5ml
Cold 30mM H_2O_2	5ml	5ml
0.01m $KMnO_4$	7ml	7ml

Peroxidase assay: The assay was based on the method of Chance and Maehly (1955) in which 0.4ml of the sample homogenate was added into clean test tubes, followed by the addition of 5ml phosphate buffer and then 5ml of hydrogen peroxide which was subsequently followed by 3ml of distilled water. Finally, the addition of 5ml of pyrogallol and the absorbance was taken at 430nm. The blank was prepared by the addition of 0.45ml of phosphate buffer, followed by 5ml of hydrogen peroxide. 3ml of distilled water was then added and finally, pyrogallol which was used to zero the spectrophotometer before taking the absorbance of the test.

Protocol:

Tubes	Blank	Test
Sample	-	0.40ml
Phosphate buffer	5ml	5ml
H_2O_2	5ml	5ml
20mM pyrogallol	5ml	5ml

Malonyldialdehyde (MDA) assay: Lipid peroxidation was estimated in terms of thiobarbituric acid reactive species (TBARS), using Malonyldialdehyde (MDA) as standard by the method of Buege and Aust (1978). 1.0ml of the sample extract was added with 2.0ml of the TCA-TBA-HCL reagent (15% (w/v) TCA, 0.375% (w/v) TBA and 0.25N HCL). The contents were boiled for 15 minutes, cooled and centrifuged at 10,000g to remove precipitate. The absorbance was read at 535 nm and malonyldialdehyde concentration of the sample was calculated using extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$.

Glucose 6-phosphate dehydrogenase (G6-PDH) assay: The assay method of Hess *et al.* (1958) was adopted in which the enzyme activity is determined by the measurement of the rate of absorbance change at 340nm due to the reduction of NAP^+. Aliquots of the homogenate supernatant (15µl) were added into test tubes while the blank contained 15µl distilled water. The reaction was initiated by adding sequentially, at fixed interval, 1000µl of reagent R1 followed by the addition of 30µl of reagent R2 and mixed thoroughly and incubated for 5 min at 37°C. The test samples and the blank were taken one at a time, and 15µl of reagent R3 and 15µl of reagent R4 were added and mixed twice by inversion and absorbance read at 340nm within 0–3 min. The spectrophotometer was zeroed with distilled water and the activity of the enzyme was estimated.

Protocol:

Reagents	Test
Sample	15µl
Reagent R1	1000µl
Reagent R2	15µl
Mix, incubate for 5mins at 37°C, then add	
Reagent R3	15µl
Reagent R4	15µl
Mix, read initial absorbance and start timer simultaneously. Read again after 1, 2, and 3 minutes	

Lactate dehydrogenase (LDH) assay: The assay method of Weisshaar *et al.* (1975) in which the enzyme activity is determined by the measurement of the rate of absorbance change at 340nm due to the reduction of $NADH^+$ was adopted. Aliquots of the homogenate supernatant (0.02ml) were added into test tubes while the blank contained 0.02ml of distilled water. The reaction was initiated by adding sequentially at fixed interval, 1.0ml of the reagent and absorbance read at 340nm within 0–3 mins. The spectrophotometer was zeroed with distilled water and the activity of the enzyme was estimated.

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Protocol:

Reagents	Test
Sample	0.02ml
Reagent R1	1.0ml
Mix, read initial absorbance and start timer simultaneously. Read again after 1, 2, and 3 minutes	

Statistical analysis: The results were calculated using mean and standard error of mean (SEM) respectively. The results from the various assays were analyzed using one way ANOVA test and for significant level of ($P<0.05$) as taken below variables. The graphical summary of the observation of efavirenz on the oxidative stress parameters on the tissues are presented below.

Results

The results obtained were calculated and the bar charts of the oxidative stress parameters on the superior colliculus and lateral geniculate body are presented below. On the tissues tested, MDA and SOD were consistent. MDA as a non-enzyme biomarker of oxidative stress is higher on the treated tissues with statistical significant ($P<0.05$) difference observed. SOD is consistently lower in the treated tissues with statistical significant ($P<0.05$) difference observed.

Superoxide Dismutase Activity (SOD) (Units/ml)

Brain tissue	Superior colliculus (SC)	Lateral geniculate body (LGB)
Control	*4.26 ± 0.04	*4.68 ± 0.13
EFV	*1.35 ± 0.04	*0.97 ± 0.05

* significant ($P < 0.05$)

From the result above, it can be seen that the SOD activity showed a significant ($P<0.05$) decreased in all the tissue under investigations as compared to the control group. The treated LGB showed an SOD decrease activity of approximately 5 fold as compared to the control group. The SOD activities of the treated SC was significantly ($P<0.05$) decreased (reflecting about 3 folds respectively) as compared to the control group.

Catalase Activity (Units/ml)

Brain tissue	Superior colliculus (SC)	Lateral geniculate body (LGB)
Control	*0.021 ± 0.000	0.019 ± 0.000
EFV	*0.010 ± 0.001	0.017 ± 0.001

* significant ($P < 0.05$)

Considering the result above, the catalase activity of the EFV treated LGB was not statistically significantly ($P<0.05$) as compared to the control group. While the catalase activity of the EFV treated SC was significantly ($P<0.05$) reduced as compared to the control group.

Glutathione Peroxidase (GP_X) (Units/ml)

Brain tissue	Superior colliculus (SC)	Lateral geniculate body (LGB)
Control	*0.775 ± 0.015	*0.715 ± 0.005
EFV	*0.655 ± 0.005	*0.840 ± 0.010

* significant ($P < 0.05$)

From the result above, the GP_x activities showed a significant ($P<0.05$) increased in the EFV treated LGB as compared to the control group respectively. While the GP_x in the EFV treated SC had a significantly ($P<0.05$) decreased activities as compared to the control group.

Malonyldialdehyde (MDA) (x10^{-5} Units/ml)

Brain tissue	Superior colliculus (SC)	Lateral geniculate body (LGB)
Control	*0.945 ± 0.001	*0.937 ± 0.002
EFV	*1.050 ± 0.002	*1.107 ± 0.003

* significant (P < 0.05)

From the result above, the MDA level showed a significant (P<0.05) increased in the EFV treated LGB and SC as compared to the control group respectively.

Glucose 6-Phosphate Dehydrogenase (G-6-PDH) Activity (nm/min)

Brain tissue	Superior colliculus (SC)	Lateral geniculate body (LGB)
Control	0.0020 ± 0.000	*0.0050 ± 0.001
EFV	0.0035 ± 0.001	*0.0025 ± 0.001

* significant (P < 0.05)

From the result above, there was a significant (P<0.05) decreased in the G-6-PDH activity in the EFV treated LGB and no significant (P<0.05) changes in the activities of the G-6-PDH between the treated efavirenz SC and that of the control group in this experiment.

Lactate Dehydrogenase (LDH) (Units/ml)

Brain tissue	Superior colliculus (SC)	Lateral geniculate body (LGB)
Control	*0.26 ± 0.04	*0.68 ± 0.13
EFV	*0.35 ± 0.04	*0.97 ± 0.05

* significant (P < 0.05)

The LDH activities in the EFV treated LGB and SC were significantly (P<0.05) increased as compared to the control group respectively.

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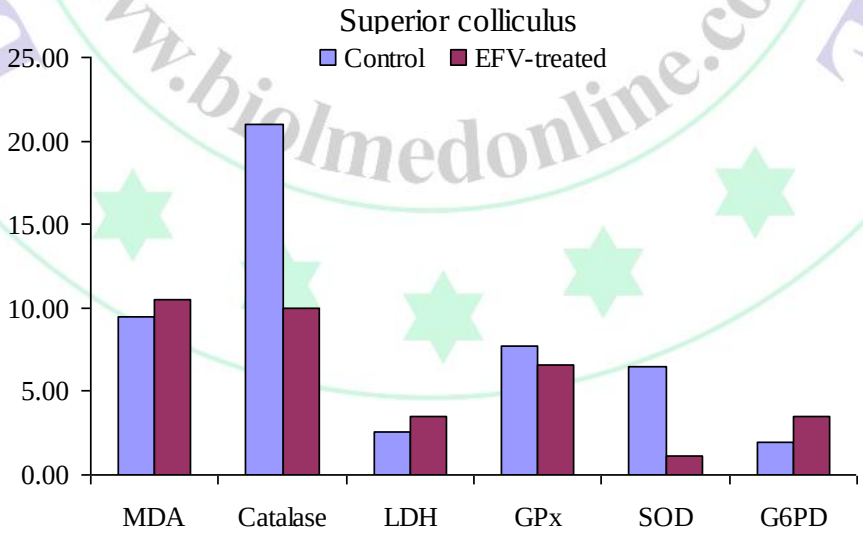

Figure 1: Bar chart showing the biochemical assays on the superior colliculus of the animals.

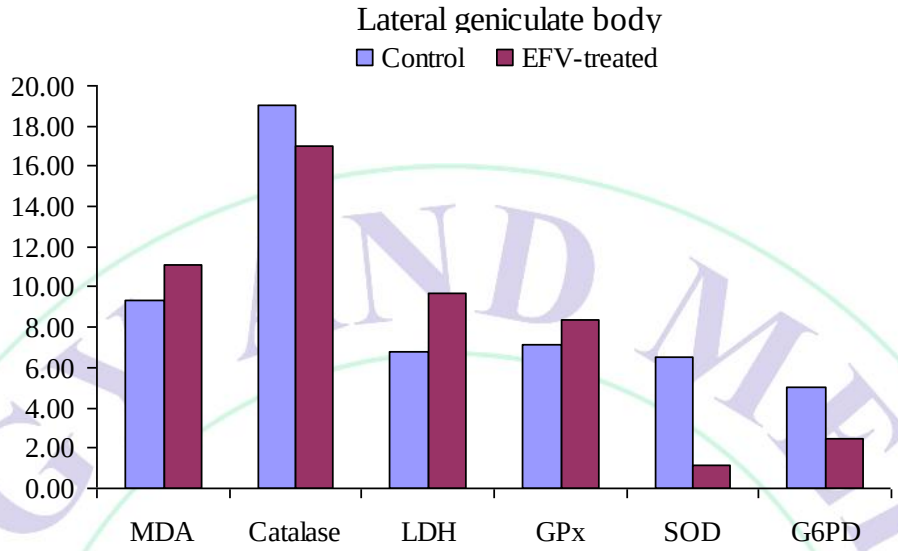

Figure 2: Bar chart showing the biochemical assays on the lateral geniculate body of the animals.

Discussion

Efavirenz has been reported to be associated with a lot of health problems in the body including the common central nervous system. Common of such problems are confusion, insomnia, abnormal vivid dreams, dizziness and headache (Jena *et al.*, 2009; Clifford *et al.*, 2005; Kontorinis and Dieterich, 2003; Yoshimoto *et al.*, 2004). It has been suggested that the toxic effects of efavirenz may be at the micro-anatomical level of the superior colliculi and lateral geniculate body of the brain (Adjene *et al.*, 2010; Adjene and Momah, 2010). However, the underlying mechanism involved in the toxicity is not well understood. This study aimed to investigate the possible role of oxidative stress in efavirenz neurotoxicity of adult Wistar rats. Most of the psychosocial and pharmacotherapeutic knowledge for the associated treatment of efavirenz toxicities especially insomnia attenuate stress (Wilson and Nutt, 2007). It was hypothesized that oxidative stress may be a biochemical mechanism of the toxicity. Thus, oxidant biomarkers (MDA and SOD) and antioxidant enzymes have been implicated in these investigations.

The results show that oxidative stress measured as MDA was found to be statistically significantly increased ($p < 0.05$) in all the treated tissues as compared with their parallel controls. Concomitantly, the activity of SOD was found to be statistically significantly decreased ($p < 0.05$) in the treated tissues as compared with their controls. The results demonstrated that efavirenz exerts its toxic effect by promoting oxidative stress in the superior colliculus and lateral geniculate body of adult Wistar rats. The observation reported here is in consonance with the studies carried out by Otitoju *et al.* (2008). They had investigated the possibility of oxidative mechanism in insecticide toxicity and reported that the oxidative stress increased in all the experimental groups (Otitoju *et al.*, 2008).

The antioxidants level is lowered while pro-oxidant polyunsaturated fatty acids are higher in the central nervous system relative to other tissues. Therefore, the central nervous system is exceptionally at risk of oxidative damage. The antioxidant enzymes such as catalase, glutathione peroxidase, G6-PDH, LDH and SOD are responsible for the brain's basic functions, both physical and cognitive (Augustyniak *et al.*, 2005; Ninfali *et al.*, 2001; Lorenzo, 1999). It is known that the activities of antioxidant enzymes are significantly modified in the central nervous system during intoxication, whereby a decrease in activity may indicate oxidative modification of the enzymatic proteins. Other viable explanations to decreased activity in an antioxidant enzyme include decrease in the synthesis rate. In this study, Catalase, G6-

PDH, GPx and LDH have presented inconsistent levels across the superior colliculus and lateral geniculate body of adult Wistar rats as demonstrated in this experiment. The increase in the activity of the antioxidant enzymes may often be due to adaptive response to excess free radicals. Furthermore, catalase may be unreliable in this respect because the decrease in its activity may be due to enhancement of protein synthesis as a confounding factor (Augustyniak *et al.*, 2005). There is also a confounding factor on G6-PDH whereby it is capable of inactivating the enzyme (Ninfali *et al.*, 2001). These confounding factors may explain the inconsistency in the relative level of these enzymes that has been observed in this study.

This study has not accessed the actual biotransformation pathways or the oxidative stress reaction pathways involved in the efavirenz toxicity. It is known that the metabolic products of efavirenz include hydroxylated products and their *O*-glucuronide, *N*-glucuronide and sulphate conjugates amongst others (Mutlib *et al.*, 1999). It is recommended that further studies aimed at performing a review of the metabolic products of efavirenz and to determine those that have pro-oxidant properties, with a view to researching how to manage the oxidative damage in efavirenz therapy.

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