Arch. Bas. App. Med. 8 (2020) 55 – 60
www.archivesbamui.com

Research Article

Diethylnitrosamine Induced Oxidative Stress and Morphological Changes in the Cerebellum of Wistar Rat

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Received: February 2019; Accepted: October, 2019

Abstract
Diethylnitrosamine (DEN), a known toxin and potent carcinogen induces oxidative stress by generation of free radicals resulting in cellular injury through its metabolized end product. There is dearth of information on DEN neurotoxicity. This study therefore, evaluated the oxidative damage and morphological changes induced by DEN in the cerebellum of Wistar rat.
Twenty male Wistar rats weighing between 110 and 120 g were divided into two groups (n=10). Group I rats received distilled water and served as the control group, while group II rats received 25 mg/kg body weight of DEN i.p. twice weekly for 12 weeks. At the end of the administration, the rats from both groups were weighed and killed. The brains were weighed and the cerebella dissected out, some preserved in phosphate buffered saline for oxidative stress and antioxidant markers, while others fixed in 10% formol-saline for histological and immunohistochemical (Glial fibrillary acidic protein, GFAP) studies, for cerebellar cytoarchitecture and astrocytes population, respectively. Data were analyzed employing the unpaired student's t-test at $p<0.05$. There was a significant decrease in weight gain in the DEN-treated group, increased lipid peroxidation, decreased glutathione levels, superoxide dismutase activity and compared with the control rats at $p<0.05$. Histological and histomorphometric evaluation of the cerebellar cortex of DEN-treated rats showed pyknosis of the Purkinje cells with chromatolysis, significant reduction in the diameter/size and loss of the Purkinje cells compared with the control rats. Immunohistochemically, there was increased population of astrocytes in the DEN-treated rats compared with the control rats. The results of the study shows that prolonged exposure of rats to diethylnitrosamine induced oxidative stress with morphological alterations in the cerebellum and thus increasing the literature of diethylnitrosamine neurotoxicity.

Key Words: Diethylnitrosamine, Oxidative stress, Neurotoxicity, Morphological changes, Cerebellar cortex

INTRODUCTION

Diethylnitrosamine (DEN) is a known toxin and potent environmental carcinogen that is present in air, water, and soil (Brown, 1999; Mittal *et al.*, 2006). It is a representative chemical carcinogen with the potential to cause tumors in various organs, including the liver, kidney, skin, gastrointestinal tract, respiratory system and at lower incidences, in other organs such as the brain (Schuller, 1992; Poirier and Beland, 1994; Verna *et al*., 1996; Park *et al*., 2009). DEN belongs to nitrosamine group, which has been recognized to cause toxicity in human beings. Diethylnitrosamine is present in different types of food products, including dairy products, soybeans, processed fish and meat, tobacco smoke and alcoholic drinks (Liao *et al*., 2001; Sivaramakrishnan *et al.*, 2008). Nitrosamines are formed endogenously from nitrate and nitrite under certain conditions such as the strong acidic pH of the stomach (Archer, 1989; Jakszyn and Gonzalez, 2006). Both environmental and food born N-nitrosamines pose a health risk for human and animals. Diethylnitrosamine has been known to induce oxidative stress and cellular damage through the release of free radicals, which are produced during the metabolic biotransformation of DEN by cytochrome P450 enzymes and form reactive electrophiles (Sheweita *et al*., 2008; Lopez-Novoa *et al*., 2011; Sheweita *et al*., 2014; Moustafa *et al*., 2017). These highly reactive free radicals initiate lipid peroxidation of the cell membrane of the endoplasmic reticulum and cause a chain reaction which can oxidize DNA, proteins and lipids (Archer, 1989; Vitaglione *et al*., 2004). Also, DEN decreased the activity and gene expression of glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD) and catalase (CAT), and the level of reduced glutathione (GSH) and increased the level of malondialdehyde (MDA) (Bendong *et al*., 2012; Kadasa and Abdallah, 2015). Oxidative stress enhances carcinogenesis through several mechanisms like injury of DNA, lipid and protein, deviations in the signaling pathways and in the expression of gene (Bansal *et al*., 2005).

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The brain, particularly the cerebrum, cerebellum and hippocampus have been reported to be highly vulnerable to neurotoxins and developmental irregularities. In the brain, oxidative stress can cause neuronal cell death, shocking brain injury, ischaemia and diseases like Alzheimer or Parkinson (Maier and Chan 2002). Research on the cerebellar cortex has been particularly intense probably due to its function in controlling various motor activities, presumable responsible for planning movement and adapting to special conditions as well as being involved in storing memories over various periods of time (Gray *et al*., 1993; Attwell *et al*., 2002). Although, DEN is aqueous soluble, it is found in lipid fractions and distributed in adipose tissue in-vivo (Gray *et al*., 1984; Pylypiw *et al*., 1984; Tricker *et al*., 1985). Diethylnitrosamine causes lipid peroxidation (Chuang and Hu, 2006) indicating potentials for penetrating and/or disrupting the blood-brain barrier, increasing brain oxidative stress with the resultant neurotoxicity. The hepatotoxicity and nephrotoxicity of diethylnitrosamine (DEN) are well documented in literatures, but DEN neurotoxicity has been addressed in only a few studies prompting the need to investigate the level of oxidative stress and morphological changes in rat cerebellum following prolonged exposure to diethylnitrosamine.

MATERIALS AND METHODS

Chemicals: Diethylnitrosamine (DEN) was obtained from Sigma-Aldrich, USA and was diluted with distilled water to concentration of 25 mg/kg body weight corresponding to approximately $1/10_{th}$ of the Intraperitoneal (rat) LD_{50}: 216 mg/kg LD_{50} of the toxicant.

Animals: Twenty male Wistar rats weighing 110-120 g obtained from the animal house of the Central animal house of the Faculty of Basic Medical Sciences, University of Ibadan were used for the study. The rats were randomly divided into two groups (groups I and II) of ten rats per group, kept in a highly ventilated and naturally illuminated (12 hour light/dark cycle) animal house of the Department of Veterinary Physiology, University of Ibadan with *ad libitum* access to food and water. The rats were allowed to acclimatize for two weeks and weighed before the commencement of treatment. All animals received human care according to criteria outlined in the Guide for the Care and Use of Laboratory Animals (prepared by the National Academy of Science and published by the National Institutes of Health).

Grouping and experimental design

Group I (control rats): Received distilled water orally with oral gavage.
Group II (experimental rats): Received 25 mg/kg/body weight of DEN intraperitoneally twice weekly for 12 weeks.

The rats were weighed, sacrificed 24 hours after the last administration and the brain of the rats dissected out. While, some cerebella preserved in phosphate buffered saline (PBS) at a pH 7.4 and temperature of 4_oC for oxidative stress evaluation, others were fixed in 10% formol-saline for histological and histomorphometric studies.

Body weight: Body weight of animals using Mettler analytical balance and percentage weight gain/loss of animals in all groups calculated as:

$$\frac{\text{Final weight (g)} - \text{Initial weight (g)}}{\text{Initial weight (g)}} \times 100$$

Oxidative stress and antioxidant markers: The cerebella of the control and experimental rats were homogenized in eight volumes of 50 mM of Tris-Hcl buffer (pH 7.4) containing 1.15% potassium chloride and the homogenate was centrifuged at 10,000 × g for 15 minutes at 4ºC. The supernatant was collected for biochemical assays of the following markers;

Lipid peroxidation (LPO) was determined by measuring the formation of thiobarbituric acid reactive substances (TBARS) present in the tissue and quantified as malondialdehyde (MDA) employing the method described by Varshney and Kale (1990). Lipid peroxidation was expressed as micromoles MDA/mg tissue (μmol/mg tissue).

Reduced glutathione (GSH) was determined according to Jollow *et al*. (1974) and expressed as μg/ml/mg tissue.
Superoxide dismutase (SOD) activity was determined by the method of Misra and Fridovich (1972) and expressed as unit/mg tissue.
Glutathione peroxidase (GPx) activity was determined by the method of Rotruck *et al*. (1973) and expressed as μg/mg tissue.

Histological, immuno-histochemical and histo-morphometric evaluations: Cerebellar tissues were processed employing routine paraffin embedding techniques and stained with Haematoxylin and Eosin (H & E) for histological and histomorphometric evaluations.
i. Histological changes in cerebellar cortex using a 500 pixel Leica digital binocular microscope.
ii. Immunohistochemical study using the anti-glial fibrillary acidic protein (GFAP) antibody and employing the Avidin biotin immunoperoxidase method to evaluate the astrocyte population in the cerebellar cortex. Briefly, sections were deparaffinized in xylene, hydrated through descending grades of ethanol and washed for 5 min in distilled water. After treatment at room temperature for 10 min with 3% hydrogen peroxide to inhibit endogenous peroxidase activity and washed in phosphate buffered saline (PBS), sections were incubated for 1 h at 37°C with anti-GFAP antibody (mouse monoclonal antibody 1:100 dilution, Leica Biosystems Inc. Ilinois, USA). Thereafter, sections were washed 3 times in PBS for 5 min each. The sections were washed in 3 changes of PBS for 5 min each, incubated with horseradish peroxidase (HRP) secondary biotinlated anti-mouse antibodies and washed in 3 changes of PBS for 5 mins. The sections were then incubated with diaminobenzidine (DAB) for 3 to 5 min and counterstained with Haematoxylin solution for 2 min and washed in running tap water (bluing) briefly. Sections were dehydrated in ascending grades of alcohol, cleared in xylene and mounted in DPX. Images were captured from the cerebellar cortex with a 500-pixel Leica binocular microscope.
iii. Histomorphometry: Thickness of the molecular layer (ML), density and diameter/size of the Purkinje cells (Pc) of cerebellar cortex using a 500 pixel Leica digital binocular microscope.

Statistical analysis

The data obtained were further analyses employing unpaired Student's t-test using GraphPad prism 6.0 version and

expressed as mean ± SEM, with the level of significance set at p< 0.05.

RESULTS

Body weight: There was a decreased weight gain in the DEN-treated rats compared with the control rats at p<0.05 (Table 1).

Oxidative stress marker and antioxidants: A significant increase in LPO, decreased GSH levels and SOD activity (p<0.05), and a non-significant decrease in GPx (p>0.05) was observed in the DEN-treated group compared with the control group (Table 2).

Histomorphometric studies: Histologically, the cerebellar cortex of the control and DEN-treated groups showed the three-layered cytoarchitecture (molecular, Purkinje and granule cell layers) containing the different neurons (Plate 1).

Thickness of the Molecular layer: There was no significant difference in the thickness of the molecular layer of the cerebellar cortex in the control and DEN-treated rats at p>0.05 (Figure 1, Plate 1).

Purkinje cell count: A significant decrease in the Purkinje cell density in the DEN-treated rats compared with the control rats at p<0.05 (Figure 2, Plate 1).

Purkinje cell diameter/size
A significant reduction in the diameter/size of the Purkinje cell was observed in the DEN-treated rat cerebellum compared with the control rats at p<0.05 (Figure 3).

Astrocyte population
There was significant increase in astrocyte population in the cerebellar cortex of the DEN-

Thickness of the Molecular layer: There was no significant difference in the thickness of the molecular layer of the cerebellar cortex in the control and DEN-treated rats at p>0.05 (Figure 1, Plate 1).

Table 1:
Mean body weight in grams of the control and DEN-treated rats

Group	Initial weight (g)	Final weight (g)	Weight gain/loss (g)	% Weight gain/loss (g)
Control	115.50±1.55	198.94±4.03	83.41±2.48	72.20±1.60
DEN	116.92±0.99	173.83±3.60	56.90±2.61[a]	48.67±2.43[a]

Values (n=10) are expressed in grams as Mean±SEM.
DEN-Diethylnitrosamine, [a]p<0.05 vs control.

Table 2:
Oxidative stress markers in the cerebellum of the control and DEN-treated rats

Group	LPO µmol/mg tissue	GSH µg/ml tissue	SOD U/ml tissue	GPx µg/mg tissue
Control	0.21±0.03	12.71±0.67	97.01±3.11	2.33±0.18
DEN	0.33±0.04a	9.42±0.84[a]	80.79±4.67[a]	1.97±0.14

Values (n=5) are expressed in grams as Mean±SEM. DEN-Diethylnitrosamine, LPO- Lipid peroxidation, GSH-Glutathione, SOD-Superoxide dismutase, GPx-Glutathione peroxidase. [a]p<0.05 vs control.

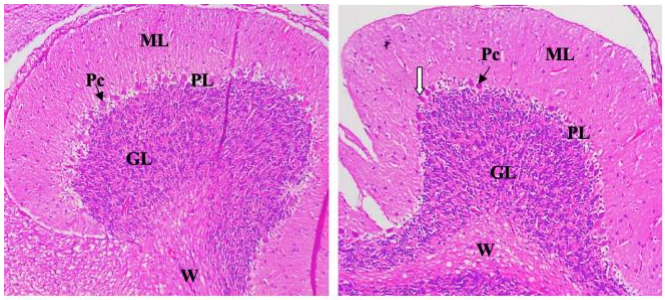

Plate 1: Photomicrographs of the cerebellar cortex of rats. a) Control, b) DEN-treated showing pyknotic (*white arrow*) decreased diameter/size and depleted Purkinje cells. DEN-Diethylnitrosamine, ML-molecular layer, PL-Purkinje layer, GL-Granule layer, W-White matter, Pc-Purkinje cell. H & E X100.

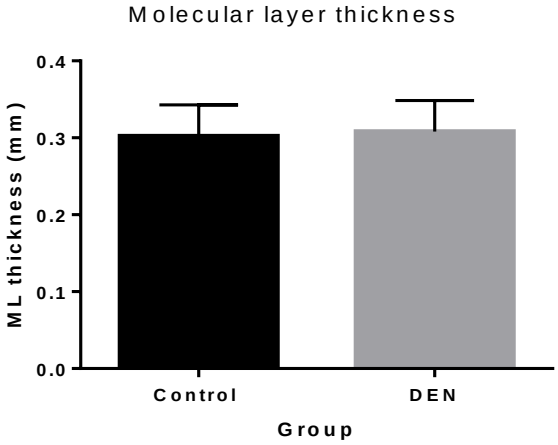

Figure 1:
Thickness of the molecular layer of the cerebellar cortex of the control and DEN-treated rats. Values (n=10) are presented as Mean±SEM. ML-Molecular layer, DEN=Diethylnitrosamine.

Purkinje cell count: A significant decrease in the Purkinje cell density in the DEN-treated rats compared with the control rats at p<0.05 (Figure 2, Plate 1).

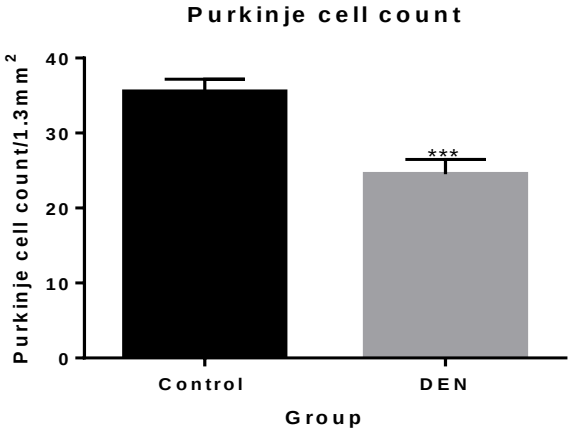

Figure 2:
Purkinje cell count/1.3mm2 area of the cerebellar cortex of the control and DEN-treated rats. Values (n=10) are presented as Mean±SEM. DEN=Diethylnitrosamine. ***p<0.01 vs control.

Purkinje cell diameter/size: A significant reduction in the diameter/size of the Purkinje cell was observed in the DEN-treated rat cerebellum compared with the control rats at p<0.05 (Figure 3).

Astrocyte population: There was significant increase in astrocyte population in the cerebellar cortex of the DEN-treated rats compared with the control at $p<0.05$ (Figure 4, Plate 2).

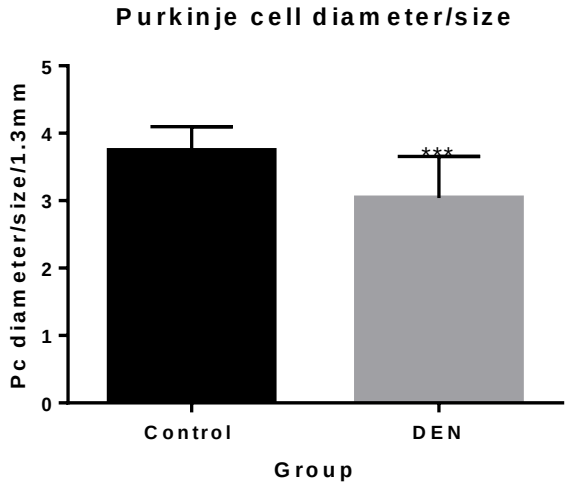

Figure 3:
Purkinje cell diameter/size /1.3mm length of the cerebellar cortex of the control and DEN-treated rats. Values (n=10) are presented as Mean±SEM. Pc-Purkinje cell, DEN=Diethylnitrosamine. ***p<0.05 vs control.

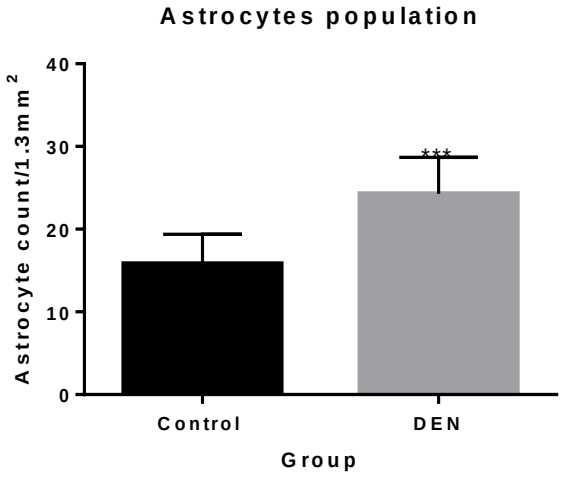

Figure 3:
Astrocyte population/1.3mm2 area of the cerebellar cortex of the control and DEN-treated rats. Values (n=10) are presented as Mean±SEM. DEN=Diethylnitrosamine. ***p<0.05 vs control.

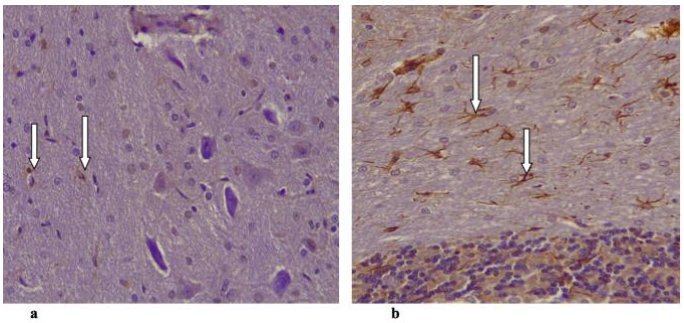

Plate 2:
Photomicrographs of the cerebellar cortex of rats. a) Control (*arrow*). b) DEN-treated with increased astrocyte population (*arrow*). DEN-Diethylnitrosamine. GFAP X400.

DISCUSSION

Diethylnitrosamine (DEN) is a representative chemical of a family of carcinogenic N-nitroso compounds found at workplaces, in processed meats, tobacco smoke, and whiskey (Schuller, 1992; Poirier and Beland, 1994; Verna *et al*., 1996). In the present study, DEN administered to rats for 12 weeks, caused a significant decrease in the body weight gain compared with the control rats. This decrease may be due to loss of appetite and reduced food intake in the DEN-treated rats, as well as changes in the energy metabolism. Ha *et al*. (2001) reported marked loss of appetite resulting in decreased mean body weight of rats treated with DEN.

Low level exposure to nitrosamine compound, commonly present in processed food has been reported to cause neurodegeneration (De la Monte and Tong, 2009). DEN induces toxicity by the generation of reactive oxygen species (ROS) resulting in oxidative stress and causing cellular dysfunction (Sheweita and Sheikh, 2011). In this study, DEN treatment increased lipid peroxidation in the cerebellum of rats accompanied by a concomitant decrease in the GSH and GPx level, and SOD activity. The observed decrease in the GSH, GPx levels and SOD activity in the present study may reflect an increased demand for these antioxidants by the cell possibly to combat ROS generation during DEN metabolism. Thus, significantly lower antioxidant levels would further aggravate the toxic effects of DEN. This finding is in agreement with the reports of Sheweita and Sheikh (2011) and Elguindya *et al*. (2018) that DEN administered to rats increased the LPO and decreased the antioxidant contents in the brain, suggesting that increased oxidative stress with failure of antioxidant defense system to stop the release of unnecessary free radicals causes tissue injury.

Histologically, the cerebellar cortex of the control and DEN-treated rats in the present study, showed the normal three-layered cerebellar cytoarchitecture, including, from the surface; Molecular, Purkinje and Granule layers. The molecular layer is the most superficial layer, containing two types of cells (outer stellate and basket), dendritic aborizations and numerous thin axons coursing parallel to the long axis of the folia (Snell, 2001). The thickness of the molecular layer of the cerebellar cortex is dependent on the amount of cells and fibres present (Rakic and Sidman, 1970). Histomorphometrically, our present study, showed no significant difference in the thickness of the molecular layer of the DEN-treated rats. The reason for the unaltered thickness of the molecular layer of the DEN-treated rats is not clear, Manto (2012) however, reported that the molecular layer is less cellular and as such, the least susceptible to chemical toxins and drugs.

The Purkinje cells are the only efferent neurons of the cerebellar cortex, exerting an inductive influence on the development of the granule cells, as well as playing a role in development and maturation of the cerebellum. As such, they are the most likely to be vulnerable to toxins (Fonnum and Lock, 2000). In this present study, there was shrinkage, chromatolysis, depletion and reduction in diameter/size of the Purkinje cells in the DEN-treated rats. The mechanisms for the above observations are not completely understood but oxidative stress has been shown to cause cellular irregularities and neuronal degeneration. Kim (2003) reported that activated nitrosamines generate reactive oxygen species thereby

increasing oxidative stress, DNA damage and lipid peroxidation, while de la Monte and Tong (2009) reported a dose-dependent decreases in mitochondrial function and ATP production, increased DNA damage and oxidative stress in post-mitotic CNS neuronal cultures briefly exposed to DEN. Also, the radical ion accumulation has been reported to impair oxidative metabolism, mitochondrial function, ATP production, and neuronal cell survival (West, 2000).

Astrocytes are specialized neuroglial cells found in the entire CNS and involved in many complex functions in healthy CNS, such as providing structural and functional support for neurons (Şovrea and Boşca, 2013), regulating the communication between already formed synaptic connections (Ota *et al*., 2013), participating in the control of brain homeostasis and the intrinsic brain defense system (Kettenmann and Verkhratsky, 2011). Astrocytes respond to all forms of CNS insults by a process called reactive astrogliosis, which has become a pathological hallmark of CNS structural lesions (Sofroniew and Vinters, 2010). Glial fibrillary acidic protein immunohistochemical method was used to display GFAP-immunoreactive astrocytes. Our present study, showed an increase in immunoreactive astrocytes in the DEN-treated rats compared with the control rats. The reason for this increase is not completely understood, however, DEN has been reported to exert its toxicity by the generation of free radicals causing oxidative stress and resulting in cellular dysfunction (Elguindya *et al*., 2018). Activation of astrocytes could result in extensive astrogliosis (Zaaraoui *et al*., 2008; Gudi *et al*., 2009) and increased reactive astrocytes found in the DEN-treated rats are indication that they are responding to CNS injuries (Eng *et al*., 2000).

From the study, prolonged administration of diethylnitrosamine rats induced oxidative stress and morphological alterations in the cerebellum by increased lipid peroxidation, decreased glutathione levels, superoxide dismutase activity, glutathione peroxidase level, Purkinje cell density and diameter, and increased astrocyte population. The above alterations are likely to interfere with normal cerebellar functions and thus increasing the literature of diethylnitrosamine neurotoxicity.

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