RESEARCH ARTICLE

Effects of N-Ethyl-N-Nitrosourea in Mice Brain in Time Fashion

Priyatosh Nath, Snehashis Modak, Tamanna Aktar, Debasish Maiti*

Abstract

Antioxidant enzyme plays a pivotal role in preventing oxidative stress. Chemical toxicants often exert adverse effects in the biological system by breaking the antioxidant response system leading to severe oxidative stress. N-Ethyl-N-Nitrosourea (ENU) is a DNA alkylating agent and is carcinogenic and neurotoxic in animals. ENU in the nervous system cause persistent alkylation of DNA within the neurons altering the normal functional activities of the brain. Prenatal exposure to ENU in rodents that generate malignant gliomas in the brain induces functional changes and apoptotic death in neuronal progenitors in the brain's subventricular zone (SVZ). However, as chemical toxicants, their role in the induction of oxidative stress in brain tissue and subsequent change in the brain were less studied. In this study, we have investigated the response antioxidant system and induction of oxidative stress in the Balb/c mice brain at various time points after ENU exposure. Exposures to ENU were found to raise the oxidative stress and lipid peroxidation in the brain of mice with subsequent reduction in antioxidant enzyme activity. A significant increase in lactate dehydrogenase activity in the serum and brain homogenate was recorded at 16th weeks after ENU injection, which indicates metabolic alteration due to damage in the brain and has been validated by histological damage in the cerebral cortex and abnormal neurologic behavior in animals administered with ENU.

Keywords: Antioxidant enzyme, N-Ethyl-N-Nitrosourea (ENU), Neurotoxicity, Oxidative stress.

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Introduction

ENU is a potent toxin, a DNA alkylating agent, and a carcinogen. The toxic effects of ENU include DNA alkylation, carbamoylation of amino acid and induction of oxidative stress.[1,2] This toxic molecule has excellent bioavailability, enabling its easy spread throughout the body tissues, blood, and other body fluids. The toxicity of ENU is not limited only to the body tissues having a direct blood supply; rather, it can cross the blood-brain barrier (BBB) to affect brain tissues. In the brain tissue, its neurotoxic effect is well established.[3] ENU in the nervous system cause persistent alkylation of DNA within the neurons. DNA alkylation causes mispairing and mutations resulting in altered gene expression and activation of carcinogenesis-related signaling pathways.[4] Trans-placental administration of ENU causes brain tumors in newborn mice and this is used as a standard procedure in experimental neuro-oncology studies.[5] Prenatal exposure to ENU generates malignant gliomas in the brain and induces functional changes and apoptotic death in neuronal progenitors in the brain's subventricular zone (SVZ).[6] However, exceptionally postnatal exposure of ENU in mice fails to develop brain tumors, although other toxicity-induced changes were seen.[7] Capilla-Gonzalez and colleagues have reported that, like prenatal exposure, postnatal exposure to ENU in mice disrupts the SVZ and diminishes the proliferative rate of neural stem cells.[8] Later, this research group reported disruption of adult neurogenesis in SVZ and dentate gyrus (DG) with strong behavioral impairments.[9] Numerous scientific reports have associated neuronal damage with ROS and oxidative stress. An alteration of normal oxidative stress and antioxidant capacity in the brain are involved with neuropsychiatric diseases.[10] A high level of ROS production was associated with various neurological disorders like Parkinson's disease or Alzheimer's disease, brain trauma to cerebral ischemia, and psychiatric disorders like autism, hyperactivity disorder, depression, schizophrenia etc.[11-14] However, ENU as an inducer of ROS and oxidative stress, its effects in brain tissues was less studied. Cellular exposures to ENU change the redox-sensitive signaling and initiate oxidative stress by altering the concentration of pro-oxidant and antioxidant enzymes in body tissues. ENU administration has been reported to reduce the activity of superoxide dismutase (SOD) and catalase enzymes.[15] The liver and kidneys of ENU exposed mice show a high level of toxicity markers SGPT, SGOT, ALP, and increased serum urea.[16] Therefore, the neuronal damage seen in adult mice on exposure to ENU might have some connection with ROS and oxidative stress. In this study, our primary focus is to check the level of various antioxidant enzymes in the brain tissues of mice at various time points after exposure to ENU and various structural changes in the cerebral cortex.

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Materials and Methods

Experimental Design and Ethical Clearance

A time point experiment was designed to study the effect of carcinogen ENU in brain of mice. This experiment included two groups of Balb/C mice named as Treated (received ENU treatment) and Control (received only PBS). Necessary clearance for the *in-vivo* experiment was taken from the institutional animal ethical committee (IAEC), Tripura University.

Housing and Maintenance of Animals

Balb/c mice about 2 weeks old were procured from Chakraborty Enterprise, Kotkata. All the animals were housed at Tripura University Animal House, maintaining the ideal living conditions, temperature and humidity, 12/12 hour light/dark cycle, and acess to a balanced diet and drink supplying all the vital nutrients.

Injecting ENU in Animals

After 2 weeks of housing the animals and careful observation of their health the treated group of mice were given 2 injection of ENU (100 mg/Kg body weight) intraperitoneal with 1 week interval. The control group of mice received 2 injections of sterile PBS instead of ENU solution.

Health Status Assessment

All the animals of ENU treated group was carefully observed for any minor changes in activities like food intake, movement, abnormal behavior, and death unless they have been sacrificed as per the below-mentioned schedule.

Sacrifice Schedule and Sample Collection

To meet the experimental goal Balb/C mice were grouped into two (Treated and Control) keeping a sufficient number of animals in each group. The day of first ENU injection was considered as day 1 and counting from that day 6 animals were sacrificed from both the groups consecutively on 4, 8, 12, 16 and 20 weeks. In the euthanized animals head was dissected carefully for collection of the brain. Collected brain tissues were processed as per the experimental need.

Estimation of Antioxidant Enzyme Activity in Brain

Superoxide Dismutase (SOD) Activity

SOD activity in brain tissue was measured according to the protocol of Beauchamp and Fridovich 1971 with little modification.[17] In brief, 3 ml of reaction mixture consisted of 50mM phosphate buffer pH-7.8, 10mM methionine, 168µM NBT, 0.025% Triton X 100 and 1.17 µM riboflavin mixed with 0.1 mL enzymatic preparation from brain tissue exposed to light emitted from a 20W fluorescent lamp in a closed box for 15 minutes. SOD inhibits photochemical reduction of NBT into formazan which was monitored at 560 nm in UV 1900 Shimadzu UV visible double beam spectrophotometer. The amount of methionine-mediated formazan formed in the presence of enzymatic preparation was compared with the amount of formazan formed in the absence of enzyme.

Catalase Activity

Activity of catalase in brain tissue was measured with little modification of the protocol of Aebi 1974.[18] For measurement 1.9 mL of 50 mM phosphate buffer, pH-7.0 was mixed with 0.1-mL enzymatic preparation in a quartz cuvette. Reaction was started by adding 1 ml freshly prepared 30 mM H_2O_2. Change in OD was then measured at 240 nm of wavelength against blank prepared by adding 1-mL of phosphate buffer instead of H_2O_2 in UV 1900 Shimadzu UV visible double beam spectrophotometer.

Glutathione Peroxidase (GPx) Activity

GPx activity was measured with little modification of the method proposed by Paglia and Valentine 1967.[19] In a quartz tube 2.9 mL of reaction mixture was taken consisting of 50 mM phosphate buffer, 1 mM GSH, 0.2 mM NADPH, 1 mM NaN_3, 1 mM EDTA, glutathione reductase enzyme 1 U/mL, and 0.1-mL enzymatic preparation. This mixture is incubated for 10 minutes at room temperature. Finally, 0.1-mL of 0.03 M hydroperoxide was added to start the reaction. Decrease in OD was then monitored for about 3 minutes against blank at 340 nm wavelength in UV 1900 Shimadzu UV visible double beam spectrophotometer.

Estimation of ROS Content in Brain Tissues

ROS in brain tissues was measured as per the protocol.[20] The reaction mixture consists of 100 µL of whole brain homogenate prepared with 40 mM Tris-HCl buffer (pH-7.4), 1-mL Tris-HCl buffer and 5 µL of 10 µM 2′, 7′-dichlorofluorescein diacetate (DCFDA) taken into 2 mL click lock micro centrifuge tube. This mixture was then incubated at 37°C for 30 minutes. Fluorescence intensity of the sample was then measured at excitation/emission wavelength = 485/525 nm using BioTek Synergy H1 plate reader.

Measurement of Lipid Peroxidation

Lipid peroxidation was measured by measuring malondialdehyde concentration in tissue according to Buege *et al*. 1978.[21] In brief 0.1-mL of 10% tissue homogenate prepared in KCl buffer mixed with 2 mL of TCA-TBA-HCl reagent and mixed thoroughly. This mixture was then heated for 30 minutes in a boiling water bath, cooled and centrifuged at 1000×g for 15 minutes. Absorbance of precipitate free supernatant was then measured at 535 nm against blank in UV 1900 Shimadzu UV visible double beam spectrophotometer.

Measurement of Lactate Dehydrogenase (LDH)

Total lactate dehydrogenase activity in tissue and serum samples were measured through the protocol of Wróblewski and Ladue 1955[22] and modified by Simaga *et al*. 2008.[23] Total of 3 mL assay mixture consisting of 100 mM potassium phosphate pH-7.0, pyruvate 0.096 mM, β-nicotinamide

adenine dinucleotide, reduced (NADH) 0.060 mM and 0.06 mL of enzyme sample (Supernatant of tissue homogenate or serum sample). Enzyme sample was always added at last to the reaction mixture and followed spectrophotometrically at room temperature for 3 min by measuring decrease in absorbance of NADH at 340 nm in UV 1900 Shimadzu UV visible double beam spectrophotometer.

Histological Study of Cerebral Cortex

For histological analysis, isolated brain from the animals was prepared as per Fischer *et al*. 2008 for cryosectioning.[24] In brief, tissues were fixed with 10% formalin for 48 hours and then the tissues were placed in 15%sucrose and 30% sucrose sequentially for 6-12 hours or until the tissues sinks. All these steps were done at 4°C temperature. Finally, the tissues were embedded in embedding media from Leica Biosystems. Embedded tissue blocks were then sectioned using Leica cryomicrotome system. Section thickness was maintained at 7 µm. Tissue sections adhered to the glass slide were then processed and stained with haematoxylin-eosin, mounted with DPX and visualized under high power objective in bright field microscopy using Leica Microscope.

Results

Abnormal Health Status in Mice Injected with ENU

Animals who received ENU injection were examined every day from the day of 1st ENU injection until the sacrifice to get information regarding their health status and identify the signs of neurological deficits. In the first and second week of ENU injection, mice were stressed with reduced food intake, less movement, and even a few deaths. These signs were abolished almost by fourth weeks and all the ENU-injected animals started behaving like normal control animals. The weight gain was found less in the ENU injected group than the control mice. The first signs of abnormality were seen by fifteenth week with lack of attention, reduced food intake. These signs increased with time and developed into neurological symptoms like inattention (lack of attention), bradykinesia (slow movement), and periodic impulsivity (characterized by sudden acceleration in movement and activities).

Increased Oxidative Stress and Lipid Peroxidation in Brain Tissue of ENU-injected Mice Associated with Reduced Antioxidant Enzymes

The level of reactive oxygen species (ROS) detected in brain homogenate as a measure of oxidative stress increased significantly in ENU-injected mice. More than 4 fold increase in the ROS level was seen within month after ENU injection, which reduced a little with time (Figure 1a). Increased ROS levels were associated with an accelerated rate of lipid peroxidation in the brain. The amount of lipid peroxidation product malondialdehyde measured in the brain of control and ENU-treated mice showed a significant increase in result of ENU exposure (Figure 1b). A sharp increase in the malondialdehyde level was seen the advancement of time with maximum on the 20th week after ENU injection. As oxidative stress results from the improper and inefficient response from the cellular antioxidant system, we have measured the concentration of various antioxidant enzyme concentrations in the brain of control and ENU-treated mice. The activity antioxidant enzymes SOD, catalase, and GPx measured showed a decline in activity in all the ENU-treated mice brain (Figure 2. A,b,c).

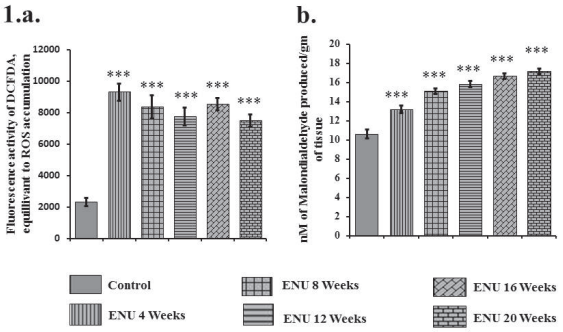

Figure 1: Showing induction of oxidative stress caused by exposure to ENU, where, **a.** ROS accumulation by DCFDA method, **b.** Malondialdehyde produced by lipid peroxidation. Values were expressed as mean ± SD. The statistical analysis was performed using oneway ANOVA followed by Tukey'smultipal comparison test in GraphPad inStat 3. Values were compared as, Control vs ENU 4 weeks, 8 weeks 12 weeks, 16 weeks, and 20 weeks. P value, $p < 0.05$ was considered as statistically significant (n = 6).I the figure, ns = Not significant, *$p < 0.05$; **$p < 0.01$;***$p < 0.001$.

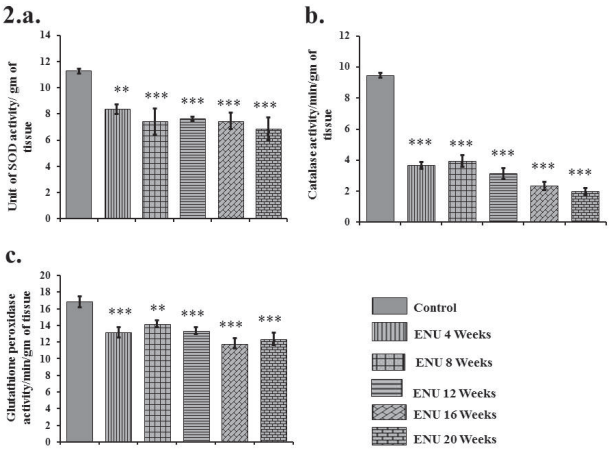

Figure 2: Showing response of antioxidant enzyme system in exposure to ENU, where, **a.** Unit of SOD activity/gm of wet tissue, **b.** Unit of catalase activity/gm of wet tissue, and **c.** Unit of GPx activity/gm of wet tissue. Values were expressed as mean ± SD. The statistical analysis was performed using oneway ANOVA followed by Tukey'smultipal comparison test in GraphPad inStat 3. Values were compared as, Control vs ENU 4 weeks, 8 weeks 12 weeks, 16 weeks, and 20 weeks. P value, $p < 0.05$ was considered as statistically significant (n = 6).I the figure, ns = Not significant, *$p < 0.05$; **$p < 0.01$;***$p < 0.001$.

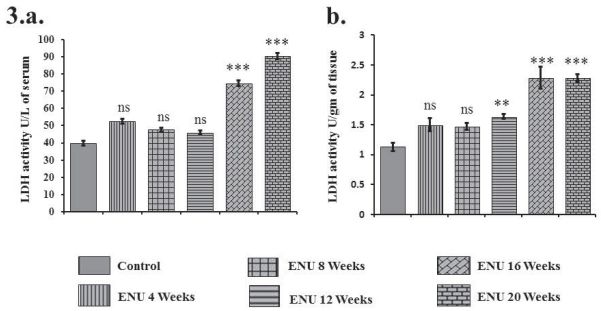

Figure 3: Showing alteration in lactate dehydrogenase (LDH) activity in exposure to ENU, where, **a.** Unit of LDH activity/L of serum **b.** Unit of LDH activity/gm of tissue. Values were expressed as mean ± SD. The statistical analysis was performed using oneway ANOVA followed by Tukey'smultipal comparison test in GraphPad inStat 3. Values were compared as, Control vs ENU 4 weeks, 8 weeks 12 weeks, 16 weeks, and 20 weeks. P value, $p < 0.05$ was considered as statistically significant (n = 6).I the figure, ns = Not significant, *$p < 0.05$; **$p < 0.01$;***$p < 0.001$.

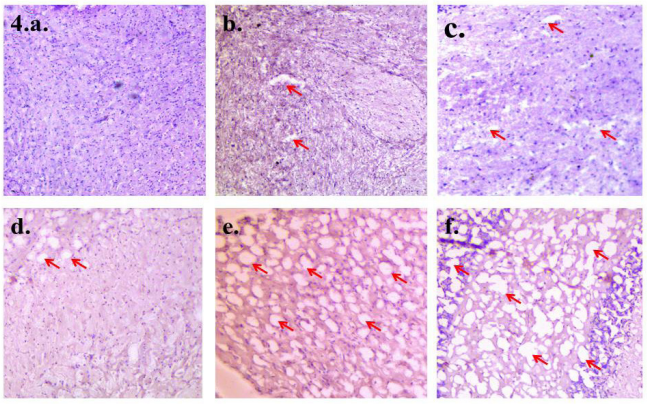

Figure 4: Showing histological changes in the cerebral cortex in exposure to ENU where, **a.** Control, **b.** ENU 4 weeks, **c.** ENU 8 weeks, **d.** ENU 12 weeks, **e.** ENU 16 weeks, **f.** ENU 20 weeks. In these figures, red arrow indicates degenerative gaps (vacuolization).

Increased Lactate Dehydrogenase Activity in Serum and Brain Tissue of ENU-injected Mice

Lactate dehydrogenase (LDH) is an important enzyme of the anaerobic metabolic pathway catalyzes the conversion of lactate to pyruvate. Change in LDH activity can be seen in multiple pathological conditions associated toxicity to structural damage. The activity of LDH measured was found to be increased in both the serum as well as in the brain homogenate of ENU injected mice. A mild to moderate increase in LDH activity seen in 4th, 8th and 12th week became significant in 16th and 20th week after ENU treatment (figure 3. A, b). The raise in LDH activity in serum may have association with blood profile as ENU also induces leukaemia in mice. The data related to leukemia induction will be reported elsewhere.

Structural Damage in Cerebral Cortex

The histological section of brain studied revealed change in granular appearance and structural abnormality in the cerebral cortex of ENU treated mice. The cerebral cortex of ENU injected mice developed degenerative gaps within the tissue in 4th week of ENU exposure is indicative of neuronal damage. These degenerative gaps (vacuolization) indicated by red arrows increased with time and are found maximum in 20 weeks, but were totally absent from control mice which received no ENU (Figure 4. a, b, c, d, e, f).

Discussion

The reactive oxygen species (ROS) has both beneficial and deleterious effects. In the nervous system, low to moderate amount of ROS are required for proper neuronal development and functions like long term potentiation (LTP) of hippocampal cells.[25] The brain tissue, being rich in lipid content (poly unsaturated fatty acids) and because of its high oxygen consumption rate is highly susceptible to oxidative stress. In normal conditions production of ROS in brain is highly regulated by its oxygen consumption and redox generation capacity and any excess of ROS is potentially neutralized by antioxidant enzyme system.But this homeostasis is severely disturbed by the presence of toxicant molecules. It is evident from the studies that chemical toxicants impair the antioxidant capacity in murine brain through altered expression of antioxidant enzymes.[27,28] A nitrosourea compound N-methyl N-nitrosourea (MNU) injected in mice alters neuro-behavior and causes enhanced production of ROS in brain tissues.[29] In this study, the carcinogen ENU used is a similar class of chemical agent as MNU. ENU is well known for its toxicity in the biological system. The interaction of ENU with intracellular macromolecules like DNA and protein causes alkylation of DNA bases to DNA adduct formation and acylation of amino acids, respectively. This can seriously break the internal homeostasis and aids in the production of excess of free radicals. In this study, we have seen significant increase in ROS upon exposure to ENU. This increase in ROS remains almost static for 5 months after first ENU injection. Production of ROS has some direct relation with antioxidant enzymes of the antioxidant response system. The antioxidant enzyme functions to neutralize or scavenge free radicals produced within the cell. A deficient antioxidant enzyme function is thus associated with increased load of free radicals and enhanced oxidative stress.[30] We, therefore, determined the activity of various antioxidants enzymes in the brain tissues control and ENU-treated mice. A reduction in the activity of superoxide dismutase (SOD), catalase, glutathione peroxidase (GSH-Px) in the brain tissue of ENU injected mice was observed.

When ROS production exceeds the scavenging capacity of antioxidant response system, extensive protein oxidation, lipid peroxidation and oxidative damage all the cellular biomolecules occurs. Oxidative stress is often described as a self-propagating phenomenon that further increases ROS production. Particularly in the brain tissue, its high fat content, high energy demand and comparatively weak antioxidant capacity makes it an easy target for oxidative insult.[31] Finally, this enhanced production of ROS causes

neuronal degeneration and functional decline in brain and other body tissues.[32]

Lactate dehydrogenase (LDH) is an important enzyme of the anaerobic metabolic pathway, particularly when the tissue has less access to oxygen due to structural damage. This enzyme converts lactate, produced in the anaerobic metabolism of glucose to pyruvate. Activity of LDH changes in multiple conditions like cancer, liver and respiratory diseases, metabolic diseases, chemical intoxications, stress, and other conditions.[33] High LDH levels have also been reported to be associated with neuronal damage.[34,35] The observed increase in LDH activity in the brain of ENU injected mice in our study therefore indicative of structural damages. The peak of LDH activity seen during the 16th and 20th week's remarks maximum of structural damage in brain caused by ENU.

Similar has been reflected in our histological study of cerebral cortex. With advancement of time after ENU injection the cerebral cortex showed signs of neuronal damage. We have seen degenerative gaps within the tissue indicative of neuronal damage. These degenerative gaps (vacuolization) increased with time and are found maximum for 20 weeks, but were totally absent from control mice that have not received ENU. Singla and colleagues has reported similar change in histoarchitecture of mice brain with alteration of lipid profile upon exposure to a similar class of compound MNU.[29] Mice also showed many behavioural changes which have developed slowly with advancement of time are indicative of functional damage to neuronal cells.[8,9] The most commonly seen behavioural changes include reduced intake of food and drink and less movement, indicative of locomotor damage. Behavioral changes are accepted as good indicators of neurotoxicity and may be observed in the initial stages of exposure to toxic compounds.[36] In our study, the behavioural changes seen mostly after months indicate chronic damage to the brain tissue by ENU.

Conclusion

This study we conclude the chronic toxicity of ENU in brain tissues. Exposure to ENU causes degeneration of neuronal structures, which may be linked to the excessive production of free radicals and oxidative stress caused by the interaction of ENU with intracellular macromolecules. The ROS, MDA and activity of various antioxidant enzymes we have measured at various time points in mice brain after ENU exposure indicate almost constant levels of oxidative stress throughout the 20 weeks study period but with a significant difference from the brains of control animals. A gene-level study would be required to further establish this relation between oxidative stress and neuronal damage at a controlled experimental set up.

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