

EFFECT OF POSTNATAL EXPOSURE TO N-NITROSODIETHYLAMINE ON THE MYENTERIC PLEXUS OF RAT

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ABSTRACT

Background: Humans are continuously exposed to the different types of nitrosamines found in the diet, drinking water, tobacco smoking, and work place. These are the potential source of exposure in the present population. Nitrosamines are found mainly in cured meat products, smoked preserved foods, beer, whiskey, pickled and salty preserved food materials. Nitrosamines have cytotoxic, carcinogenic and mutagenic properties. Nitrosamines exert toxic or mutagenic effects by promoting DNA damage, oxidative stress and reactive oxygen species formation that causes increased lipid peroxidation, adduct formation, and pro-inflammatory cytokine activation. Increased chronic exposure of low doses of nitrosamines is unavoidable in current environmental conditions. The nitrosamine explored in this study is N-Nitrosodiethylamine (NDEA), representing environmentally significant nitrosamine.

Materials and Methods: The present study was conducted on pups of wistar rats, (*Rattus norvegicus*). Six pregnant wistar rats having same pregnancy time were taken. After delivery sixteen pups were chosen randomly. The control and the experimental groups had eight pups each. Sterile water and NDEA were given as 0.2mg/kg intraperitonea daily to the control and the experimental groups of rat pups respectively, from postnatal day 1 to postnatal day 20. All the rat pups were sacrificed on postnatal day 21 to obtain the tissues of the gastrointestinal tract.

Results: A significant reduction of morphometric parameters such as the area, the perimeter and the ferret diameter of the perikaryon of the myenteric neurons of the experimental group found. The number of the myenteric neurons per unit area of muscularis externa was also significantly reduced in the NDEA treated wistar rat pups.

Conclusions: Chronic low-level exposure of N-Nitrosodiethylamine (NDEA) caused significant effect on the histoarchitecture of myenteric plexus of wistar rats.

KEY WORDS: N-Nitrosodiethylamine (NDEA), Enteric Nervous System, Myenteric Plexus, NADPH-Diaphorase, Wistar Rat.

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BACKGROUND

Earlier studies explored only the carcinogenic, mutagenic and teratogenic properties of N-Nitrosodiethylamine (NDEA) amine. Recently it had been reported that chronic exposure of low-

level, sub-mutagenic doses of NDEA also poses a great threat to humans and other mammals. Some researchers documented that chronic low doses of NDEA exposure which is present in different food materials and environment may

be one of the causations of increased incidence of insulin resistance diseases in the present changing life styles [1].

N-Nitrosodiethylamine is a nitrosamine compound that is a slightly yellow, volatile liquid at room temperature. It is soluble in water, ethanol, ether, organic solvents, and lipids. Physical and chemical properties of N-nitrosodiethylamine are- molecular wt. 102.14, specific gravity- 0.9422 at 20°C/4°C, Melting point-<25°C, Boiling point- 175°C- 177°C, Water solubility- 106gm/L at 24°C [2].

Ming tong et al., [1,3] suggested that N-Nitrosodiethylamine (NDEA) in chronic low doses created a serious health problems to human kind. Environmental and food contaminant exposures to chronic low, sub-mutagenic levels of nitrosamines, promote major insulin resistance diseases including type 2 diabetes mellitus, non-alcoholic steatohepatitis, and alzheimer's disease. Nitrosamines are responsible for increased DNA damage and generation of reactive oxygen species. The increased productions of reactive oxygen species result in enhanced lipid peroxidation, protein adduct formation, and pro-inflammatory cytokine activation [1,3]. Few earlier studies showed marked histopathological abnormalities in the hippocampal formation in the temporal lobe with severe neuronal loss, indicating a profound effect of N-Nitrosodiethylamine on neuronal tissues of the central nervous system. N-nitrosodiethylamine (NDEA) was injected in Long Evans rat pups intraperitoneal for three alternative days to generate in vivo model for studying NDEA toxicity. In the present study we designed our own in vivo model to investigate the effect of NDEA on the myenteric plexus of wistar rats.

The autonomic division of the peripheral nervous system (PNS) consists of three subdivisions: the sympathetic, the parasympathetic and the enteric nervous system (ENS). The ENS is the largest and most complex sub-division of the PNS. It is linked to the central nervous system (CNS) through the parasympathetic and the sympathetic nervous system however it can also function independently. The enteric plexuses contain intrinsic primary afferent neurons and

interneurons that enable the ENS to mediate integrative responses to local stimuli independently. It is also called as a 'second brain' of vertebrates. Its study is important in neuro-gastroenterology. It contains different types of plexuses, special cells, neurotransmitters etc. It controls the local blood flow, muscular activity, mucosal transport, secretions, immune modulation and endocrine functions etc.

The ENS is composed of a vast number of neurons and glial cells. These cells are clustered into multiple interconnected ganglia embedded within the gut wall. The enteric ganglia are organized into two major plexi: the myenteric (or Auerbach's) plexus and the submucosal (or Meissner's) plexus. The myenteric plexus is situated between the longitudinal and circular smooth muscle layers and it runs along the full length of the gastrointestinal tract. The submucosal plexus is closely associated with the connective tissue, between the circular muscle and the innermost mucosal tissue. The size of enteric ganglia is variable between myenteric and submucosal ganglia in the gut regions and also among species. Myenteric ganglia are larger than submucosal ganglia in the same region of the gut.

MATERIALS AND METHODS

The present study was conducted on pups of Wistar rats, (*Rattus norvegicus*). Six healthy pregnant female wistar rats having same pregnancy time were procured from the Central Animal Facility (CAF) of AIIMS, New Delhi, after obtaining ethical clearance from Institute Ethical Committee (IACE 676/12) and kept under observation. The rats weighed 180-200 gm (pregnant rats) at the time of commencement of the study. The day of delivery of pups was considered as postnatal day zero. The litters along with the dams were confined in cages, kept in temperature (20-26°C) and humidity (30-70%) controlled environment. The animals received standard rodent diet and normal tap water ad libitum. All procedures for the care and use of laboratory animals were carried out in accordance with the principles laid down by Institute Ethical Committee.

N-Nitrosodiethylamine (NDEA) used in the prese-

present study was purchased from Sigma-Aldrich (India). LD50 of NDEA in adult wistar rat has been reported 216mg/kg intraperitoneal [2]. In the present study NDEA was administered in a dosage of 0.2 mg/kg equivalent to 0.09 % of LD50 respectively. A standard stock solution of NDEA was prepared by dissolving 1gm of NDEA (liquid) in 2lt of sterile water to make 0.5mg/ml solution. de la Monte et al., [3] used NDEA as 2mg/kg intraperitoneal for three alternative days in long Evans rat pups and saw the effect on hippocampus. We established 0.2mg/ kg daily intraperitoneal dose of NDEA for 20 days. All the doses were 50 to 100 fold lower than the cumulative doses used to cause cancer [1,4]. NDEA (0.2mg/kg) was administered by intraperitoneal injection to experimental rat pups with the help of Hamilton micro syringe from postnatal day 1 to postnatal day 20. The intraperitoneal route was chosen to ensure that the pups receive the exact dosage of the chemical. The control group received double distilled pyrogen free sterile water by the same route. During the treatment period, the animals were weighed daily and observed constantly for general features of well being. On the day of sacrifice i.e. post-natal day 21, the pups were anesthetized with ether in air tight container. The pups were then perfusion fixed. The gastrointestinal tissues were dissected out carefully and stored in 4% paraformaldehyde at 40°C till further processing.

Gastrointestinal tissues from all the rat pups were fixed in fresh 4% buffered paraformaldehyde for 2 hours at 4°C. They were washed thoroughly after fixation in chilled 0.1 M phosphate buffer cryo-protected in 15% and then in 30% sucrose at 4°C for 3 hours and 8 hours (till tissue sinks) respectively. The samples were frozen in OCT (optimum cutting temperature) compound (Tissue Tek) and 20 μ thick systemic random serial sections were cut and every ten section were taken using a cryostat (Leica). Frozen sections were mounted onto 1% gelatin coated slides (1% w/v gelatin, 0.01% w/v chromium sulphate Cr (SO₄)₂.12H₂O) and air dried. Slides were kept at -20°C for further enzyme histochemistry. NADPH-diaphorase histochemistry stained discrete populations of enteric neurons. Cryostat sections on the glass

slides were washed several times with 0.1 M phosphate buffer (pH 7.4). NADPH diaphorase activity was visible by incubating the sections in 10 ml 0.1 M Tris- Cl buffer (pH 7.8, adjusted with few micro-litre of concentrated HCL) containing 10mg NADPH, 1mg Nitroblue-tetrazolium (NBT) and 0.3% Triton X-100 at 37°C for 60 minutes to 90 minutes in the incubator in dark. The treatment with TritonX-100, a detergent, is required because it is a good permeabilizing agent. Reactions were terminated when the stain was sufficiently intense (60 min-90 min) by washing the tissues gently with chilled 0.1 M phosphate buffer and the sections were mounted in a mixture of glycerol and phosphate buffer (4:1) for morphological study [5]. The stained sections were examined under a microscope and images were captured using a CCD (charge-coupled-device) camera connected to a frame grabber card in an IBM PC interfaced with a Zeiss binocular microscope. The images were saved as JPEG files with minimum compression and maximum quality. The images were then analysed using ImageJ (developed at the US National Institute of health, available at <http://rsb.info.nih.gov/ij/>). Before making the measurements the system was calibrated using a micrometer scale (Carl Zeiss, Germany) for the magnification at which the images were acquired. The region of interest (cells, area, perimeter, diameter etc.) was accurately outlined using a digitizer tablet and a stylus connected to the system.

The following parameters were used for analysis:

1. Neuronal (myenteric) cells profiles (area, perimeter, ferret diameter) of perikaryon.
2. Neurons (myenteric plexus)/unit area of the muscularis externa

More than 800 NADPH diaphorase positive cell profiles were studied and well stained cells with proper perikaryon were chosen for morphometric analysis.

The SPSS software package was used for statistical analysis. Data was expressed as mean \pm standard deviation. Independent sample t-test (Student's t-test) was used to determine the statistical significance between the means. Standard error of mean (SEM) and the 95% confidence interval (CI) of the difference between

the means were noted. For all statistical tests, probability levels of less than, or equal to, 5% (two-tailed p value < 0.05) were considered to be significant.

RESULTS

NADPH-Diaphorase-positive neuronal cells and their processes were found in the myenteric and submucous plexuses of stomach, ileum and distal colon. It stained only the nitric oxide synthase (NOS) positive neurons. The myenteric neurons had a distinct pale nucleus with surrounding dark blue cytoplasm. The neuronal processes and their arborizations were also stained deep blue. The outlines of the perikaryon were clearly demarcated. The population of NADPH-diaphorase-positive neurons in the myenteric plexus was extremely heterogeneous in terms of cell size. The cells were pleomorphic in size. The myenteric neurons were more in numbers and larger in size as compare to experimental groups. Non-neuronal cells such as endothelial, mast cell, smooth muscle cell, glial cells and mucosal epitheliocytes etc., did not take up the stain. But the four layers of the gut could be clearly distinguished.

Morphometry of diaphorase positive neurons: Estimation of the myenteric neuronal cell size (area, perimeter and ferret diameter) of diaphorase positive perikaryon and number of neurons per 5 mm² of muscularis externa of the stomach, ileum and distal colon were conducted using imageJ package.

Effects of postnatal NDEA treatment on the stomach morphometry: Results showed that the NDEA treatment during postnatal period led to decrease in the mean area, perimeter and ferret diameter; (Fig: 1, 2; Table: 1), of perikaryon of nitreergic myenteric neurons of stomach in comparison to the control (Fig: 1, 2; Table: 1). Postnatal NDEA treatment led to significant reduction in the NADPH- diaphorase positive myenteric neurons/ 5mm² of the muscularis externa of the stomach as compared to the control (Fig: 3; Table: 2).

Effects of postnatal NDEA treatment on the ileal morphometry: Results showed that the NDEA treatment during postnatal period lead to decrease in the mean area, perimeter and ferret diameter; (Fig: 4, 5; Table: 3) of perikaryon of nitreergic myenteric neurons of ileum in comparison to the control (Fig: 4, 5; Table: 3).

Fig. 1: Cross section (20µm) of wistar rat pups stomach in NADPH- Diaphorase stain at postnatal day 21 (Group; A, B). Fewer and smaller size myenteric neurons (black arrow) and submucosal neurons (red arrows) can be seen in the experimental group (B) as compared to the control group (A). LM- longitudinal muscle, CM - circular muscle, SM - submucosa. Scale bar- 50 µm.

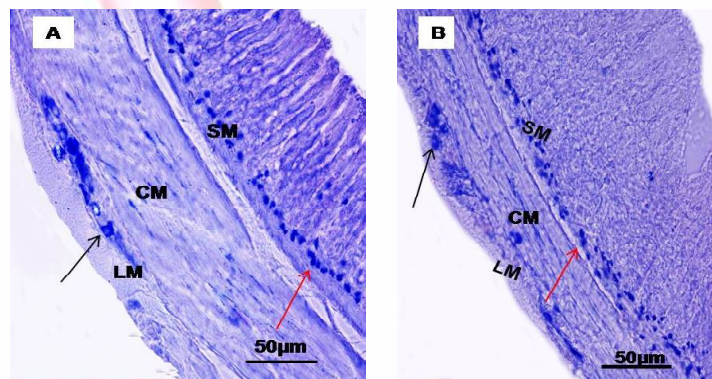
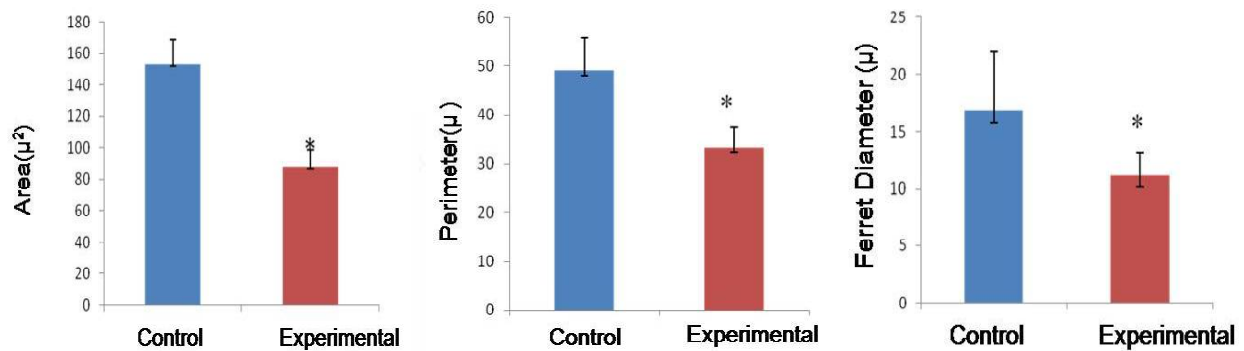


Table 1: Area, Perimeter & Ferret Diameter of NADPH – Diaphorase positive perikaryon of myenteric neurons of postnatal treated stomach of wistar rat pups sacrificed at postnatal day 21 (control & experimental).

Stomach (postnatal treatment)	Area ± SD (µ ²)	Perimeter ± SD (µ)	Diameter ± SD (µ)
Control group (A)	153.92 ± 14.18	48.95 ± 6.84	16.84 ± 4.13
Experimental group (B)	85.23 ± 11.91*	33.15 ± 4.23*	11.21 ± 1.93*
P value	P < 0.0001*	P < 0.0001*	P = 0.004*

*Significant when compared to the control group (p < 0.05). All result are expressed as mean ± SD; n = 8 rat pups per group.

Fig. 2: Area, Perimeter & Ferret Diameter of NADPH- Diaphorase positive perikaryon of myenteric neurons of postnatal treated stomach of wistar rat pups (control & experimental).



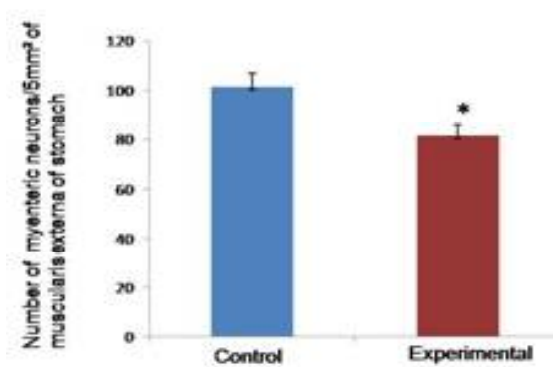
* Significant when compared to the control group (P< 0.05).

Table 2: Number of NADPH- Diaphorase positive myenteric neurons / 5mm² of muscularis externa of wistar rat pups stomach.

	Stomach Group A (control)	Stomach Group B (experimental)
Myenteric neurons / 5mm² ± SD	101.3 ± 5.496	81.41 ± 4.529*
P value		P < 0.0001*

*Significant when compared to the control group (P< 0.05). All result are expressed as mean ± SD; n = 8 rat pups per group.

Fig.3: Number of NADPH- Diaphorase positive myenteric neurons / 5mm² of muscularis externa of wistar rat pups stomach.



*Significant when compared to the control group (P< 0.05).

Fig. 4: Cross section (20µm) of wistar rat pups ileum in NADPH- Diaphorase stain at postnatal day 21 (Group; A, B). Fewer and smaller size myenteric neurons (black arrow) and submucosal neurons (red arrows) can be seen in the experimental group (B) as compared to the control group (A). LM- longitudinal muscle, CM - circular muscle, SM - submucosa, LP- lamina propria. Scale bar- 50µm.

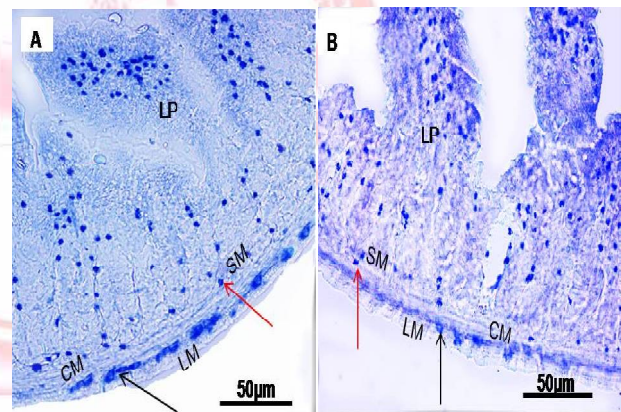


Table 4: Number of NADPH- Diaphorase positive myenteric neurons / 5mm² of muscularis externa of wistar rat pups ileum.

	Ileum Group A (control)	Ileum Group B (experimental)
Myenteric neurons / 5mm² ± SD	150.003 ± 7.422	99.116 ± 7.436*
P value		P < 0.0001*

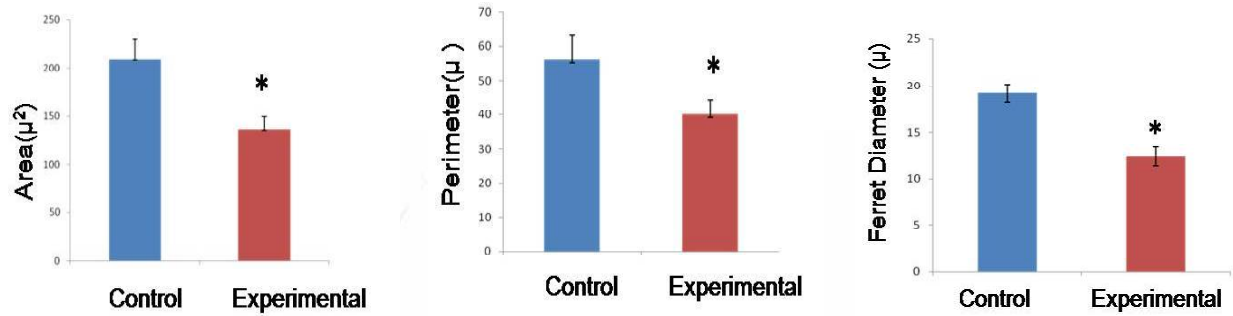
*Significant when compared to the control group (P< 0.05).

Table 3: Area, Perimeter & Ferret Diameter of NADPH – Diaphorase positive perikaryon of myenteric neurons of postnatal treated ileum of wistar rat pups sacrificed at postnatal day 21 (control & experimental).

Ileum (postnatal treatment)	Area ± SD (µ²)	Perimeter ± SD (µ)	Diameter ± SD (µ)
Control group (A)	209.126 ± 20.237	57.065 ± 6.113	19.203 ± 3.801
Experimental group (B)	136.387 ± 13.352*	40.086 ± 4.44*	12.405 ± 2.661*
P value	P< 0.0001*	P< 0.0001*	P= 0.001*

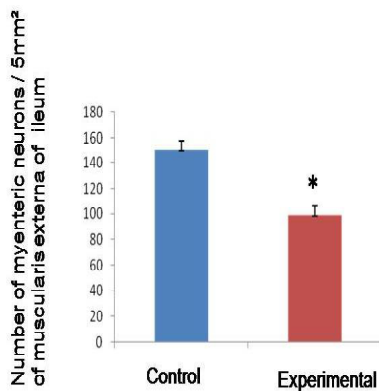
*Significant when compared to the control group (p< 0.05). All result are expressed as mean ± SD; n = 8 rat pups per group.

Fig. 5: Area, Perimeter & Ferret Diameter of NADPH- Diaphorase positive perikaryon of myenteric neurons of postnatal treated ileum of wistar rat pups (control & experimental).



*Significant when compared to the control group (P< 0.05).

Fig. 6: Number of NADPH- Diaphorase positive myenteric neurons / 5mm² of muscularis externa of wistar rat pups ileum.



*Significant when compared to the control group (P< 0.05).

Fig. 7: Cross section (20µm) of wistar rat pups distal colon in NADPH- Diaphorase stain at postnatal day 21 (Group; A, B). Fewer and smaller size myenteric neurons (black arrow) and submucosal neurons (red arrows) can be seen in the experimental group (B) as compared to the control group (A). LM- longitudinal muscle, CM - circular muscle, SM - submucosa. Scale bar- 50 µm.

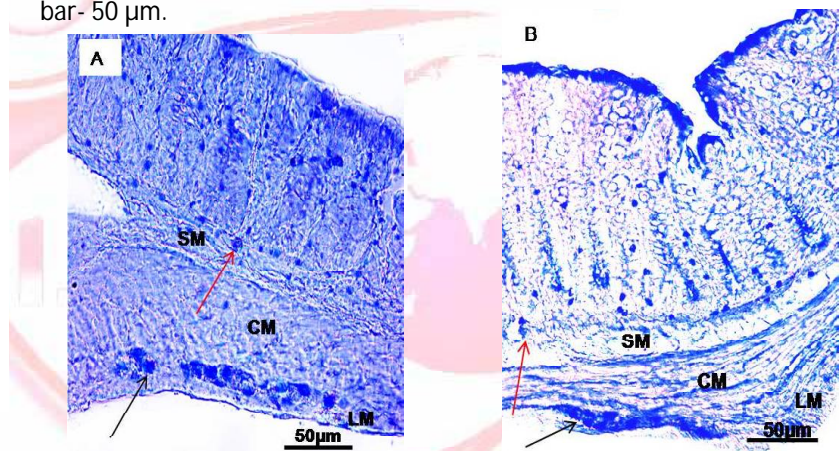
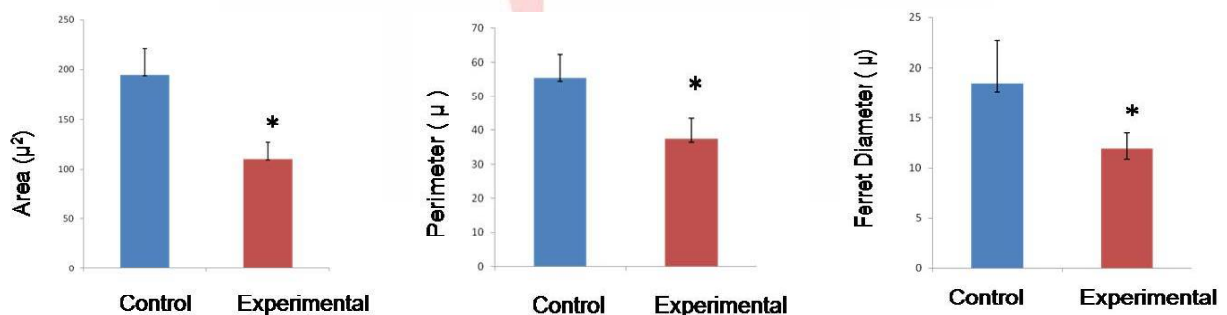


Table 5: Area, Perimeter & Ferret Diameter of NADPH – Diaphorase positive perikaryon of myenteric neurons of postnatal treated distal colon of wistar rat pups sacrificed at postnatal day 21 (control & experimental).

Distal colon (postnatal treatment)	Area ± SD (µ ²)	Perimeter ± SD (µ)	Diameter ± SD (µ)
Control group (A)	191.707 ± 26.617	54.975 ± 7.364	18.513 ± 4.243
Experimental group (B)	110.035 ± 16.689*	36.121 ± 5.77*	11.911 ± 1.605*
P value	P< 0.0001*	P< 0.0001*	P= 0.001*

*Significant when compared to the control group (p< 0.05). All result are expressed as mean ± SD; n = 8 rat pups per group.

Fig. 8: Area, Perimeter & Ferret Diameter of NADPH- Diaphorase positive perikaryon of myenteric neurons of postnatal treated distal colon of wistar rat pups (control & experimental).



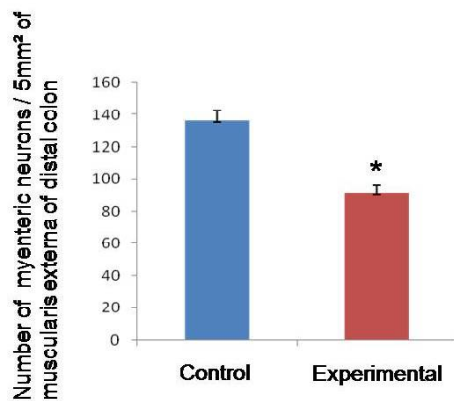
*Significant when compared to the control group (P< 0.05).

Table 6: Number of NADPH- Diaphorase positive myenteric neurons / 5mm² of muscularis externa of wistar rat pups distal colon.

	Distal colon Group A (control)	Distal colon Group B (experimental)
Myenteric neurons / 5mm ² ± SD	136.413 ± 6.053	91.396 ± 4.791*
P value		P < 0.0001*

*Significant when compared to the control group (P < 0.05).

Fig. 9: Number of NADPH- Diaphorase positive myenteric neurons / 5mm² of muscularis externa of wistar rat pups distal colon.



*Significant when compared to the control group (P < 0.05).

Postnatal NDEA treatment led to significant reduction in the NADPH-diaphorase positive myenteric neurons/ 5mm² of the muscularis externa of the ileum as compared to the control (Fig: 6; Table: 4).

Effects of postnatal NDEA treatment on the distal colon morphometry: Results showed that the NDEA treatment during postnatal period led to decrease in the mean area, perimeter and ferret diameter; (Fig: 7, 8; table: 5) of perikaryon of nitrergic myenteric neurons of distal colon in comparison to the control (Fig: 7, 8; table: 5). Postnatal NDEA treatment led to significant reduction in the NADPH-diaphorase positive myenteric neurons/ 5mm² of the muscularis externa of the distal colon as compared to the control (Fig: 9; Table: 6).

DISCUSSION

Nitrosamines are well known carcinogenic, mutagenic and teratogenic agents [2]. It is present in water, soil and air. Humans are continuously being exposed to a wide range of nitrosamines from diets, tobacco smoking, work places and drinking water [6]. Nitrosamines are

also present in cured meat products, smoked preserved foods, beer and whiskey, pickled and salty preserved foods. Nitrosamines are absorbed by skin, airways and the alimentary tract [7]. Important nitrosamines are N-Nitrosodiethylamine (NDEA), N-Nitrosodimethylamine (NDMA), and N-Nitrosopyrrolidine (NPYR) etc.

The Enteric Nervous System (ENS) is derived from the neural crest cells. It is composed of different neuronal ganglionated plexuses. The main components of which are the myenteric (Auerbach's) and the submucous (Meissner's) plexuses. The myenteric plexus is mainly concerned with motor activity of muscularis externa, and the submucous plexus with various activities of mucosa of the gut wall. Nitrosamines generally affect the gastrointestinal tract and its associated organs, and the brain [2,7].

Previous studies mainly explored the carcinogenic potential of NDEA. Changed life styles and wide distribution of nitrosamines in the environment prompted many researchers to investigate if there are any other effects of nitrosamines on mammalian biological systems, in chronic, sub-mutagenic doses. A remarkable *in vivo* model of rat pups developed by Tong et al., lead to the finding that NDEA treatment (intra-peritoneal) caused Type 2 Diabetes Mellitus, Non-Alcoholic Steatohepatitis, and neurodegeneration [1,3]. There was evidence of neuronal loss in the hippocampal formation. The final conclusion was that all these phenomena resulted from the direct effect of NDEA and increased production of hepatic ceramide (toxic lipid). Both NDEA and ceramide are lipid soluble, cross the blood brain barrier easily and affect the neuronal and glial cell survival, neurotransmitter function and synaptic plasticity.

It was well established that chronic exposure to sub-mutagenic doses of N-Nitrosodiethylamine (NDEA) has profound effect on the neuronal cells. Following these clues, the present study was designed to investigate the effects of post natal exposure to NDEA on the enteric nervous system of wistar rat pups. The dosages of NDEA were kept at 50 to 100-fold lower than the cumulative doses used to cause cancer [1,4].

CONCLUSION

The potential routes of human exposure to NDEA are ingestion, inhalation, and dermal contact. Now it is obvious that the general population may also be exposed to unknown quantities of NDEA present in foods, beverages, tobacco smoke, drinking water, and industrial pollution. The present study clearly indicated that the chronic, low-level exposure to NDEA had significant effect on the histoarchitecture of myenteric plexus of wistar rats. So the humans are also at the risk in the present scenario.

Conflicts of Interests: None

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