Original Article

Neuromorphometric Analysis of the lateral prefrontal cortex of young Wistar rats exposed to nicotine in utero

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Objective: To examined the effect of prenatal nicotine administration during neurodevelopment on morphometric parameters of neurons and the implication on neurologic functions after birth.

Methodology: Twenty female Wistar rats were time mated and grouped into two main groups (A and B), each subdivided into a Control (A_1 and B_1) and a Treatment subgroup (A_2 and B_2). Control animals received 0.1 ml normal saline, while treatment groups received 13.76 mg/kg/d nicotine intraperitoneally. Administration was for 5 consecutive days between day 9-13 of the 2nd gestational week (Group A) and day 16-20 of the 3rd gestational week (Group B). At delivery, the weights of the pups were taken and also at postnatal day 15 prior to sacrifice. Animals were anesthetized with ketamine and perfused transcardially with saline and 4% paraformaldehyde. Tissues were processed for Nissl staining using cresyl fast violet, and analyzed for histochemical and morphometric changes.

Results: Findings included low birth weights, altered brain growth pattern, altered neuronal morphology and low neuronal indices.

Conclusions: The neuronal morphological and morphometric changes observed in this study could underlie many of the neurobehavioural abnormalities seen in offspring prenatally exposed to nicotine during neurodevelopment. (Rawal Med J 2014;39:331-336).

Keywords: Neurodevelopment, lateral prefrontal cortex, prenatal nicotine.

INTRODUCTION

The use of tobacco is of serious public health concern, and one of the leading causes of preventable death.[1,2] One of the main constituents of tobacco, nicotine, is responsible for most of its effects,[3] including the additive and neurological effects. There is a geographical shift in tobacco use from the West to the developing world,[4] and a high percentage of women, including teenage girls, use tobacco or cigarette.[5] Tobacco smoking is common among pregnant women and pregnant adolescents,[6] with serious health implication on the developing fetus. Despite measures by different Governments to control tobacco use, only little success has been recorded.[7]

Nicotine is teratogenic, and responsible for the neurologic and behavioral abnormalities.[8] Prenatal nicotine exposure (PNE) is linked with development of learning disabilities, attention deficit disorders, hyperactivity and behavioral disorders in children.[9] Many of these are due to dysfunctions of the prefrontal cortex (PFC), especially the lateral prefrontal cortex (LPFC), whose functions also include attention and awareness, implementation of behavioral rules, setting multiple behavioral goals, and short-term retention of information.[10]

The processes involved in neurodevelopment include proliferation, migration, differentiation, synaptogenesis, myelination and apoptosis.[11] At each of these periods, the developing brain is vulnerable to environmental insults, which can impair the normal course of development, with resulting neurodevelopmental dysfunctions.[12] Nicotine, through maternal active or passive smoking, can affect one or more of these developmental processes.[11] The resulting neurodevelopmental deficits manifest in the offspring after birth, and could linger through adolescence to adulthood.[13] This study was designed

to determine the effects of prenatal nicotine administration during neurodevelopment on neuronal morphology and some neuromorphometric parameters, and the implication on neurologic functions after birth.

METHODOLOGY

Twenty adult female Wistar rats weighing 162.1±4.89 g were obtained, put in different cages (5 in each cage) and housed in the Animal House of the College of Health Sciences, University of Ilorin. They were fed on pelletised growers feed (UAC Vital Feeds®, Nigeria) and water liberally.

The oestrus phases of the female rats were determined using the vaginal smear technique as described by Marcondes et al.[14] Wistar rats have four identifiable oestrous phases, and the animals were grouped accordingly, though temporarily prior to mating.[14] Mature male Wistar rats were introduced to the female rats, overnight, in their proestrus phase. The presence of spermatozoa in the vagina, as examined from vaginal smear obtained the following morning, and viewed under the light microscope [14] was indicative of successful mating. This finding was presumably used as a confirmation of pregnancy, and this was noted as gestational day 0.[15]

Pregnant rats were divided into two main groups; A and B, representing the 2nd and 3rd week of gestation, respectively. Each was further subdivided into a Control (A_1 and B_1) and a Treatment (A_2 and B_2) subgroup. The 1st week of gestation was not considered in this study since neurodevelopment in rats begins in early 2nd week.[11] Nicotine was obtained from BDH Chemical Ltd. Poole, England. The treatment subgroups (A_2 and B_2) were given 13.76 mg/kg/d nicotine (slightly lower than the LD_{50} of 14.6 mg/kg),[16] in two divided doses intraperitoneally, while the control subgroups A_1 and B_1 received equal volume (0.1 ml) of normal saline. All administrations were for 5 consecutive days during the respective gestational periods, such that Group A received nicotine from days 9-13 of the 2nd gestational week and Group B from days 16-20 of the 3rd gestational week (GW). After delivery, the weights of the pups were taken and also on postnatal day (PND) 15, just before sacrifice.

At PND 15, pups from each subgroup were anesthetized with about intramuscular ketamine. After induction of anesthesia, the pups were perfused transcardially with saline, and subsequently with 4% paraformaldehyde in 0.1M phosphate buffer. After perfusion, the skull was dissected out and the brain was removed, weighed and placed in fixative for subsequent tissue processing. Tissue blocks were sectioned using a rotary microtome (Leitz Wetzlar® 1512, Germany) at a thickness of 5 μm. Tissue staining procedure employed was cresyl fast violet technique,[17] to demonstrate Nissl granules and for stereological and morphological analysis of neurons.

Stained sections were viewed using Eclipse FNI Microscope (Nikon®, Japan) attached to a Motic Camera (Moticam 2300; 3.0M pixel USB 2.0; Motic®, Honk Kong), at magnification 40x with a water interface between the objective lens and the slide sections. The diameter of cell bodies and nuclei of neurons were measured using the Motic Images Plus 2.0 ML software. The ImageJ win32 (NIH, USA) Software was used for cell counting. Counting of cells was carried out using a Counter Window of 1024 x 768 pixels dimensions (resolution); Width: 1024 pixels (160 microns); Height: 768 pixels (120 microns). For each slide preparation, five different Counter Windows were analyzed, captured at different fields of the sections, with care taken to ensure consistency in sampling, and to reflect a fair representation of the cortical layers. The average of the five Windows was determined and reported as number of cells per 160x120 μm slide area (or per Counter Window).

The findings were analysed by student's t test using SPSS version 16.0 and data are presented as Mean±SEM, with determination of level of significance, at $p<0.05$.

RESULTS

Nicotine exposure during the 2nd and 3rd weeks of gestation resulted in reduced birth weights. The low birth weights were statistically significant ($p<0.05$) in both gestational weeks, compared with their respective controls (Table 1).

Table 1: Body and Brain Weights of Pups.

Parameters	2nd GW: Control	2nd GW: Treatment	3rd GW: Control	3rd GW: Treatment
	A_1	A_2	B_1	B_2
Birth Weight (g)	5.88 ± 0.185	4.44 ± 0.117	5.73 ± 0.12 $^{\gamma}$	5.27 ± 0.11 $^{\gamma}$
Weight at PND 15 (g)	20.49 ± 0.47 ^{c}	16.54 ± 0.92 ^{c}	20.87 ± 0.42 ^{d}	16.18 ± 0.36 ^{d}
Weight Diff (g)	14.61	12.1	15.14	10.91
Brain Weight (g)	1.07 ± 0.02	1.16 ± 0.08	1.02 ± 0.03	1.01 ± 0.03
Brain-body weight ratio	0.0522	0.0701	0.0489	0.0624

Significant statistical changes between: $^{c}A_1$ and A_2; $^{d}B_1$ and B_2; $^{?}A_1$ and A_2; $^{\gamma}B_1$ and B_2 ($p<0.05$). NB: A_1 and B_1 received 0.1 ml normal saline in the 2nd and 3rd weeks of gestation respectively, while A_2 and B_2 received 13.76 mg/kg/d nicotine in the 2nd and 3rd weeks of gestation respectively.

The body weights of the young rats on PND 15 remained low in the treatment groups compared to their respective controls ($p<0.05$). However, the brain weight of rats exposed to nicotine in the 2nd GW was higher than that of the control ($p>0.05$), whereas brain weight in the treatment group of the 3rd GW was slightly lower than the control ($p>0.05$), when examined on PND 15 (Table 1).

Table 2: Measurement of nuclear and somatic diameters of neurons in the lateral prefrontal cortex.

Parameters	Groups			
	A_1	A_2	B_1	B_2
Somatic diameter (µm)	15.737 ± 0.515	14.377 ± 1.009	14.140 ± 0.682	13.897 ± 0.452
Nuclear diameter (µm)	11.823 ± 0.316 †	8.529 ± 0.467 †	11.045 ± 0.746	8.792 ± 0.517
Perinuclear-Somatic space (µm)	3.914 ± 0.289	5.848 ± 1.245	3.095 ± 0.278	5.105 ± 0.264
Nuclear-Somatic ratio	0.7513	0.5932	0.7811 $^{\neq}$	0.6327 $^{\neq}$

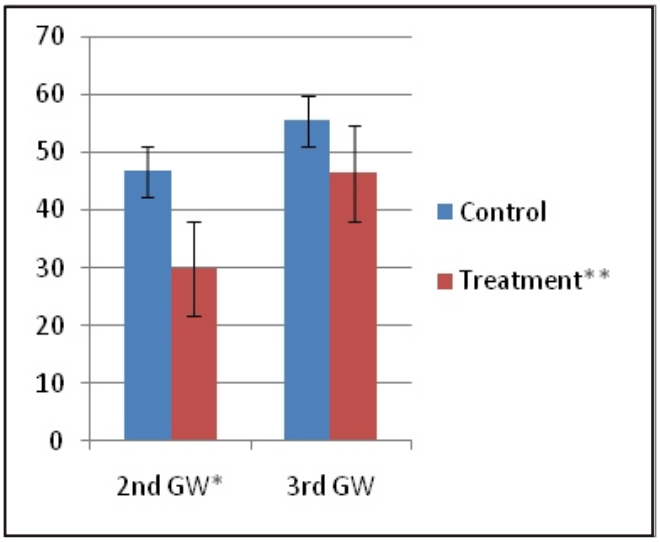
Statistically significant difference ($p < 0.05$) between † A_1 and A_2; $^{?}B_1$ and B_2.

NB: The perinuclear-somatic space is the difference between the somatic and nuclear diameter (or the interval between the soma and the nucleus), which represents the part of the cytoplasm not occupied by nucleus.

Neuronal cell count was reduced in the treatment groups, but statistically significant difference was seen only in the 2nd GW group ($p<0.05$). The severity of reduction was significantly more in rats exposed in their 2nd GW ($p<0.05$) compared to the 3rd GW exposed rats (Fig. 1).

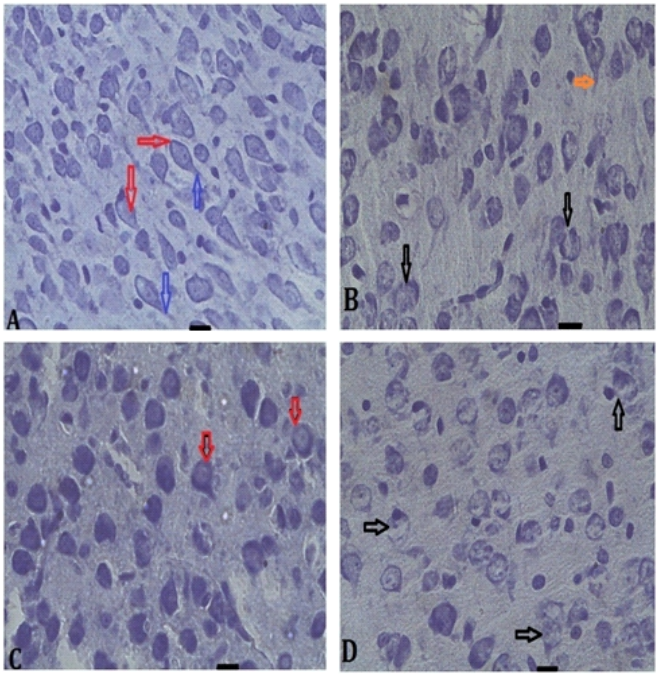
Both somatic and nuclear diameters were reduced in both treatment groups, with significant difference only in nuclear diameter of the 2nd GW group ($p<0.05$) (Table 2). The nuclear-somatic ratio was low in both treatment groups, with a significant difference in the group exposed during the 3rd GW ($p<0.05$) (Table 2).

Fig. 1: Average neuronal cells count.



The general architecture of the LPFC of the control rats was preserved, with the presence of numerous neurons and glial cells. Many of the neurons could be seen with visible parts of the axonal segments, especially the initial part. The nucleoli of the nuclei were prominent in many of the neurons. The histology of the treatment groups was distorted and did not follow a regular pattern. Fewer neurons were demonstrated and fewer also retained their initial axonal segment. Some of the neurons either degenerated or were degenerating, as there were features suggestive of damaged cells and loss of membrane integrity. The nucleoli were less prominent and there was also reduced positivity for Nissl staining compared to the control (Fig. 2).

Fig. 2: Photomicrographs of the LPFC of Control rats of the 2nd GW (A) and 3rd GW (C), and the Treatment groups exposed to nicotine in the 2nd GW (B) and 3rd GW (D). In **A and C:** there were numerous neurons (red arrows), many with preserved initial axon segment (blue arrows), and prominent nucleoli. In **B and D:** there was reduction in neuronal cells population and many appeared to be degenerating (black arrows); there were less visible axon segments (brown arrow); the nucleoli were less prominent, and the general architecture was distorted. Staining intensity for Nissl substance was also reduced in the treatment groups.



DISCUSSION

The exposure of the fetus to nicotine through maternal smoking, or environmental exposure to secondhand smoke,[18] is detrimental to the developing brain. Low birth weight was noted in the current study. Prenatal nicotine administration causes intra-uterine growth retardation and the eventual low birth weight.[3,13,19]

Nicotine is associated with low appetite, and central regulators of appetite have been described, with the presence of nicotinic receptors in the appetite regulating area of the hypothalamus.[20] These brain regulators include neuropeptide Y (NPY), which is down-regulated on exposure to maternal nicotine, thereby reducing appetite.[19] Hence, cigarette smoking during pregnancy causes intra-uterine growth restriction with severe implication on developing organs and increased risk of adverse postnatal health outcomes.[3] Some studies have reported a catch-up growth following prenatal nicotine administration, probably due to cessation of nicotine exposure after birth.[19] Such subjects could have an initial increased growth rate faster than non-exposed subjects, such that they outweigh the latter. As earlier observed,[21] nicotine cessation is associated with increased hypothalamic NPY, and a consequent increased drive to eat, and reduced capacity for energy expenditure. This could account for the higher rate of weight gain observed after birth in the nicotine-exposed rats in such studies. Body weight of treated rats in the current study remained lower than the Control rats, and growth rate was also lower in the former. Reasons for variation in observation from this study could be in relation to the dose of nicotine used, the time of exposure and the postnatal day the observation was made. However, brain weight was raised in rats exposed to nicotine during the 2nd gestational week.

The dose of nicotine used in the current study was on the high side, and activation of nicotinic acetylcholine receptors (nAChRs) has been said to adversely affect brain morphogenesis and neuronal survival in rodents, with persistent neurochemical alterations.[22] According to Slotkin,[23] nicotine stimulates nAChRs to cause mitotic arrest of brain cells. This mitotic arrest could be due to inhibition of spindle formation and severe damage to the spindle structure with consequent effect on chromosome alignment and segregation.[23] This impairs normal cell division.

Exposure to nicotine in the 2nd week of gestation at the onset of neurulation had a direct effect on the neurons. This probably disrupted the formation processes and resulted in the very low level of neuron cells formed. Meanwhile, exposure to nicotine only during the 3rd week of gestation after many neurons have been produced already led to more of structural damage rather than a numerical deficit, and the structural damage to cells was more marked in rats exposed to nicotine in the 3rd week of gestation. Features such as loss in the continuity of

membrane layers and membrane damage, with possible leakages of intracytoplasmic and intranuclear contents, and loss of cellular components, were evident.

Measurement of the diameters of the cell bodies and nuclei were used to determine their sizes. There were generalised reduction, and the nuclear diameter was markedly reduced, especially during the 2nd week of gestation. This also reflected in the nucleus-to-soma ratio. Impairment in cellular activity and cell division process would prevent optimal cell development, with the consequent formation of small-sized cells with other cellular components, such as nuclei affected.[32, 33] This might also be responsible for the less prominence of nucleoli, which would affect very important cellular processes, such as protein synthesis.

CONCLUSION

Short-term exposure to nicotine during early neurodevelopment could result in severe structural damage to neuronal cells, which perhaps is evidence reflecting neurochemical derangement within the cells arising from fetal exposure to nicotine. These changes could underlie many of the neurobehavioral and neurologic problems observed in offspring that were exposed to nicotine-containing substances during prenatal development of the brain.

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Analysis and interpretation of the data: GO Omotoso, OB Akinola
Drafting of the article: GO Omotoso
Critical revision of the article for important intellectual content:
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Conflict of Interest: None declared
Rec. Date: Mar 19, 2014 Accept Date: May 30, 2014

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