Effects of Nicotine Administration on the Structure of Auditory Cortex of Adolescent Male Guinea Pigs, a Histological and Ultrastructural Study

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ABSTRACT

Background: Nicotine, the main ingredient in tobacco smoke, has always been linked to degenerative changes to the nervous system and several areas in the brain were reported to be injured due to nicotine. The effect of nicotine on the auditory system is only being recognized recently with few studies assessed the morphology. The effect of nicotine on the primary auditory cortex of young adolescent animals was addressed in this study.

Materials and Methods: Twenty young male guinea pigs of two months old were divided into two groups of 10 animals each. Group I, the control group, received daily subcutaneous injections of normal saline for one month. Group II, the nicotine-treated group, received 3 mg/Kg body weight of nicotine subcutaneously daily. After animal sacrifice, brains were removed and processed for light and electron microscopic evaluations. Morphometry was also done to light microscopic histological sections.

Results: In the nicotine-treated group, there were degenerative changes affecting the neurons, glia as well as blood capillaries. There was a darkening of neurons and disruption of their dendrites and organelles. The glial cells revealed reactivity, swelling, and cytoplasmic disruption. Blood capillaries showed collapse and thickening of their basement membrane. Morphometry revealed that the thickness of the auditory cortex has decreased as well as the dark neuronal number has increased in the treated group versus the control.

Conclusion: Nicotine administration to adolescent male guinea pigs resulted in degenerative changes affecting the auditory cortex of the brain, which emphasizes the hazardous effects of cigarette smoking, especially at a young age.

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Key Words: Adolescence, auditory cortex, Guinea pig, nicotine, ultrastructure.

INTRODUCTION

Cigarette smoking has always been associated with damage to various organs in our body, including the brain. There are numerous ingredients in tobacco smoke, among which nicotine, has been well known to be responsible for addiction and overall hazardous effects. Chronic cigarette smoking was proposed to cause degenerative changes in brain morphology, blood flow, neurochemistry and neurophysiology[1-3]. Regarding brain structure, it was reported that human smokers had smaller volumes and density of grey matter and white matter of frontal, parietal and temporal cortices, compared to controls, which were revealed by magnetic resonant imaging[4,5]. There has been a limited information in the literature about cigarette smoking and auditory central nervous system with only evidences recognized recently. Nicotine is implicated in affecting auditory cognitive functions and was reported to enhance sensory evoked responses in animals and humans[6]. Regarding the morphologic changes affecting the auditory cortex after nicotine administration, little data is present so far. Early nicotine exposure can negatively affect the development of the nervous system including that of the auditory cortex. Studies indicate that adolescence period is very critical for adaptation and developmental changes in the brain resulting from exposure to nicotine[7]. Recently a study addressed the effect of nicotine on the morphology of young auditory cortex. Smoking during adolescence can worsen the risk of long term dependence[8]. Furthermore, exposure to nicotine during adolescence may interfere with maturation of thalamocortical area and adversely influences sensory
and cognitive functions\(^{(11)}\). Data from animal studies found out changes in the expression pattern of nAChRs in the brain during development (Reviewed in\(^{(12)}\)). Interestingly some receptors are detected at birth with peaks in the second postnatal week (this time coincides with the start of the sense of hearing) and decrease during the third week in rodents\(^{(13)}\). On the other hand other receptors increase in the second and third postnatal week\(^{(14)}\). Adolescence is the time of increased sensitivity for nicotine impact on auditory processing and higher cortical functions.

Therefore, this study aimed to address the effect of smoking on the auditory cortex morphological structure of young adolescent male guinea pigs. Layer V was chosen in this work because its cells form the major part of the projection to the inferior colliculus and cochlear nuclei of the auditory pathway\(^{(15,16)}\).

**MATERIALS AND METHODS**

**Materials**

Nicotine was used as hydrogen tartrate salt (Sigma–Aldrich (St. Louis, MO, USA). The drug was diluted in 0.9% normal saline and administered subcutaneously daily. Twenty young pigmented male guinea pigs were included in this study. They were 1 month age, weighing 300–350 gm at the beginning of the study, they were housed under laboratory standard conditions of temperature and light, as well as food and water ad libitum. The institutional ethics committee of Assiut University issued approval of the experimental design. The care and use of animals were according to the outlines of the National Institutes of Health Guidelines. The guinea pigs were divided into two groups: Group I included 10 animals and considered as the control group. They were given 0.5 ml of normal saline for one month subcutaneously daily. Group II: This group included 10 animals and given 3 mg/kg body weight of nicotine for one month subcutaneously daily.

**Methods**

At the end of the experiment, the animals were sacrificed under carbon dioxide inhalation. Then they were perfused with formalin or glutaraldehyde fixative transcardially through the left ventricle.

**Histological Study**

Light microscopy: the brains were exposed dorsally, and coronal sections were made in the primary auditory area, an area located near the pseudo Sylvian sulcus\(^{(17,10)}\). The tissue blocks were fixed in 10% neutral buffered formalin or paraffin blocks were processed and cut through auditory cortex. Then, (5–7 µm) sections were stained with hematoxylin and Eosin (Hx &E) for general orientation and morphometric evaluation. Bielschowsky’s silver method was used to study the neurofibrils in the neurons of the auditory cortex. Slides were examined and photographed using a light microscope (Axio Scope A1, Carl Zeiss Microscopy, Germany) connected to a camera. Electron microscopy: The auditory cortex was sectioned by

the help of a dissecting microscope into small specimens. Specimens were fixed in 2% glutaraldehyde and processed for transmission electron microscopy. Semi-thin sections (0.5–1 µm) were stained with toluidine blue and Layer V was defined according to\(^{(9)}\) (Elgayar \textit{et. al.}, 2016). Ultrathin sections (500– 800 Å), for the chosen parts of layer V of the primary auditory cortex in semi-thin sections, were stained with uranyl acetate and lead citrate. Ultrathin sections were studied with the transmission electron microscope JEOL (JEM_100CXII, Tokyo, Japan) and then photographs were obtained at 80 KV in Assiut University-Electron Microscope Unit. All histological studies were performed according to\(^{(9)}\).

**Morphometric Study**

The thickness of the auditory cortex was measured by the use of an image analyzer soft-ware using the light microscope (Axio Scope A1, Carl Zeiss Microscopy, Germany). The measurements were obtained by using the 5X objective lens of a light microscope. The thickness of the auditory cortex was obtained at three points using arbitrary distance method in the same axis of each section. The cortical thickness was calculated as the mean of the three readings from five non-overlapping Hx &E serial sections of two different tissues per animal. This procedure was performed in three different animals from each group. The number of (light and dark neurons) per field, was calculated from the previous sections by touch count method using the objective oil immersion lens (100X) from three different fields per section in layer V in each group. The percentage of each light and dark neuron was calculated for the total number of neurons for both groups. The morphometric measurements were performed by a researcher who was blinded to the experimental design.

**Statistical Analysis**

In the statistical analysis: SPSS Version 21 was used. The data were analyzed using independent t-test. Results were expressed as means _ SD.

**RESULTS**

The adolescent guinea pig auditory cortex was similar to adult guinea pigs as described by\(^{(16)}\). The 6 cortical layers were distinct as follows: Layer I had very few cell bodies. Layers II and III contained densely packed cells the majority of which were pyramidal. Layer IV had very packed small granule cells. Layer V had mostly pyramidial cells and was low in density. Layer VI had high density of cells than layer V and its cells were smaller than layer V (Figure 1).

**Group I**

Examination of cells in layer V of control animals revealed that most cells were pyramidal which were large pale cells with prominent nucleoli. Some glial cells could be detected which were smaller and darker than neurons. Blood capillaries were also found with their normal configuration (Figure 2).
Ultrastructural examination revealed that the pyramidal cells had euchromatic nuclei with prominent nucleoli. The cytoplasm had many RER, mitochondria, Golgi bodies and very few small lysosomes (Figure 3A and B). Dendrites could be observed with their organized structure (Figure 3B). Neuroglial cells as astrocytes, oligodendrocytes and microglia. The microglial cells were identified by their smaller size, small nucleus and large heterochromatic clumps. The surrounding cytoplasm had free ribosomes, few mitochondria, and short RER strands (Figures 3A and C). The astrocytes had round to oval bodies with scanty cytoplasm and ovoid nuclei containing peripheral and some scattered heterochromatin, surrounded by long processes (Figures 3D and E). The oligodendrocyte was identified by marginal chromatin, and electron dense cytoplasm (not illustrated). Blood capillaries could be found with their endothelium and basement membrane (Figure 3F).

**Group II**

The layer V of nicotine treated animals showed that most neurons were dark with variable staining intensity. The dark neurons were shrunken with irregular nuclei and outline (Figure 4).

Ultrastructurally, the dark neurons exhibited heterochromatic nuclei with variable cytoplasmic electron density containing ill-defined organelles, dilated RER and multiple dense bodies (Figures 5A and B). Neuronal processes revealed mild dense cytoplasm with dissociation of their microtubules, which appeared wavy (Figure 5C). Myelin sheaths of nerve fibers had shown many changes as folding and focal splitting (Figures 5D). Very few neurons had euchromatic nuclei with electrulcent cytoplasm, decreased Nissl granules, and disrupted organelles and some neuronal nuclei revealed increase in the size of their fibrillar centers (Figure 6A). Oligodendrocytes had irregular dense chromatin of their nuclei, and dense cytoplasm. Few of them had intense electron dense nuclei, scanty cytoplasm having ill-defined Golgi bodies, and free ribosomes (Figure 6B). Astrocytes revealed swelling of the cytoplasm and processes, which contained many glycogen granules and dark mitochondria (Figures 6A, B, C). Blood capillaries had irregular outline, with irregular folded nuclei, and irregular lumen. Endothelial cells were disorganized and the basal lamina was markedly thickened and irregular (Figure 6C). Microglia were mostly associated with blood capillaries. They had heterochromatic nuclei and their cytoplasm is scanty and contained dense bodies (Figure 6D).

When silver staining was done for histological sections of layer 5 of control auditory cortex it showed neurons and neuropils stained faintly brown (Figure 7A). While the layer 5 of the treated auditory cortex revealed intense staining with marked increase in fibrillar component (Figure 7B)

**Morphometry**

The mean thickness of the auditory cortex was reduced in group II (nicotine treated) versus group I (control). (Table 1 and Histogram 1). There was a significant decrease in the number and percentage of light neurons in group II (nicotine treated) compared to group I (control) (Table 2 and Histogram 2). There was a significant increase in the number and percentage of dark neurons in group II (nicotine treated) compared to group I (control) (Table 3 and Histogram 3).
Fig. 2: Histological sections through layer V of the auditory cortex of control animals A: is showing mainly light neuronal cells (N) with pale cytoplasm and prominent nucleoli. A blood capillary (Ca) is visible with a glial cell (G) nearby. B: is showing light neurons and some glial cells are also seen among neurons and processes. Toluidine blue stain X 400.

Fig. 3: Electron microscopic pictures of layer V of the auditory cortex of control animals showing different cell types. A: is showing a neuron with pale cytoplasm and euchromatic nucleus (n) with prominent nucleolus (nu), well organized Nissl bodies and organelles. A microglia (Mg) is seen near the neuron (N) (X 3600). B: exhibits part of a neuron with its cytoplasm and nucleus (n) a dendrite (D) (X 10000). C: A higher magnification of part of figure A showing a microglia with its few organelles and heterochromatic nucleus (X 10000). D: reveals an astrocyte (As) with a thin rim of cytoplasm and ovoid nucleus and. A neuron is shown with organized structure. A part of myelinated nerve fiber (M) can also be recognized (X 3600). E: A higher magnification of part of figure D to show the detailed structure of the nucleus and cytoplasm of the astrocyte (As) and neuron (N) (X 10000). F: is showing part of a neuron and part of a blood capillary with well-organized basal lamina (BL) (X 10000).
**Fig. 4:** Histological section through layer V of the auditory cortex of nicotine-treated animals. A: is showing mainly dark neuronal cells (N) with dark cytoplasm and prominent nucleoli. A blood capillary (Ca) is visible with thick wall. B: is showing dark neurons (N) and some glial (G) cells are scattered among neurons and processes. Toluidine blue stain X 400.

**Fig. 5:** Electron microscopic pictures of layer V of the auditory cortex of treated animals. A: is showing dark neuronal cells (N) with dense nuclei, and shrunken dark cytoplasm and irregular myelin sheath (M). Part of a dendrite (D) is shown (X 1900). B: is showing a dark neuron with disorganized structure and swollen astrocytic processes (star) (X 7200). C: is showing parts of dendritic processes (D) with disorganized microtubules (mt) (X 5800). D: A higher magnification of figure A showing the detailed affection of the myelin (M) which was in the form of folding and splitting of the myelin sheath, with disorganized microtubules (mt). Part of a neuron is seen with dark cytoplasm (N) (X 5800).
Fig. 6: Electron microscopic pictures of layer V of the auditory cortex of nicotine-treated animals showing different cell types. A: is showing a neuron (N) with large nucleus (n) and a prominent big nucleolus (nu) with a large fibrillar center. There are cytoplasmic dense bodies and disrupted organelles. An astrocyte (As) is observed with a scanty cytoplasm and ovoid heterochromatic nucleus (X 3600). B: is showing an oligodendrocyte (O) with dense cytoplasm. An astrocyte (As) can also be seen as well (X 5800). C: is showing a collapsed blood capillary (Ca) with thickened basal lamina (BL) and narrow lumen (Lu). Dilated astrocytic processes (Ap) rich in glycogen granules (Gl) can be seen (X 5800). D: reveals a microglial (Mg) cell with dark nucleus and scanty cytoplasm (X 5800).

Fig. 7: Histological section of layer 5 of control auditory cortex (A) showing neurons and neuropils stained faintly brown (arrow heads). While the treated group (B) are showing moderate staining (arrow heads) and intense staining (arrows). Silver stain X 400
**EFFECTS OF NICOTINE ON AUDITORY CORTEX OF ADOLESCENT GUINEA PIGS**

**Table 1: Thickness of Auditory Cortex**

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<tr>
<th></th>
<th>Group 1 (Control)</th>
<th>Group 2 (Nicotine)</th>
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<tbody>
<tr>
<td><strong>Mean</strong></td>
<td>1280.53</td>
<td>1104.64</td>
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<tr>
<td><strong>SD</strong></td>
<td>51.23</td>
<td>32.89</td>
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<tr>
<td><strong>P value</strong></td>
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*P < 0.1, **P < 0.05, ***P < 0.01

**Table 2: Light neurons and their percentage**

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<td><strong>Light cell number</strong></td>
<td>10.38</td>
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<td><strong>SD</strong></td>
<td>1.51</td>
<td>1.87</td>
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<td><strong>P value</strong></td>
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**Light cell %**

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<td><strong>Mean</strong></td>
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<td><strong>SD</strong></td>
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<td><strong>P value</strong></td>
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*P < 0.1, **P < 0.05, ***P < 0.01

**Table 3: Dark neurons and their percentage**

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<td><strong>Dark cell number</strong></td>
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**Dark cell %**

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<th>Group 2 (Nicotine)</th>
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<td><strong>Mean</strong></td>
<td>100</td>
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<td><strong>SD</strong></td>
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<td><strong>P value</strong></td>
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*P < 0.1, **P < 0.05, ***P < 0.01

**DISCUSSION**

This study demonstrated a negative association between chronic nicotine administration and the integrity of the auditory cortex in young adolescent male guinea pigs. The effects on adolescents is very important and critical because this time coincides with the typical time of the start of smoking by human subjects\[^{20,21}\]. Furthermore, this period of time includes the time of development of auditory function\[^{22,23}\]. Our findings can be correlated with changes in different regions in the brain as the temporal lobe in...
which there was decrease in the volume and density of grey matter in smokers versus nonsmokers[43]. Adolescent smoking is linked to deficits in auditory cognitive functions as well as alteration in structural changes in thalamo cortical systems, suggesting that higher auditory function is critical to nicotine administration during adolescence[22, 24]. Nicotine administration was reported to increase dendritic length in pyramidal cells of layer V of prelimbic cortex of adolescent and adult rats and this effect was different according to age[23]. Furthermore, the effects of nicotine on the neuronal size and dendritic spines of prefrontal cortex vary according to dose of nicotine[29]. The presence of nACHRs on endothelial cells may be linked to human pathology because in tobacco users, such as nACHRs are exposed to large amount of nicotine and accordingly might be activated[28]. It has been shown that the brain areas, which revealed decreased volume density in smokers, also expressed nACHRs. Now it is evident that the nACHRs are crucial components of the auditory pathway[23]. Nicotine binds to nACHRs which normally control the activities of the acetylcholine neurotransmitter in the auditory system[24].

In our study, many neurons revealed large fibrillar centers in the nucleus, which was detected by electron microscopy. In support with this finding, it was reported that fibrillar centers are the copies of nucleolar organizer regions in the interphase nucleus and the site of storage of rRNA genes[30]. The nucleus has been proposed as the controller of nucleolar functional organization and the site of transcription of rRNA and ribosome biogenesis[27, 28]. This finding may suggest that chronic nicotine administration may result in decrease in the metabolic and transcription activities of cells in layer V of the cortex. Generally cells with highly decreased metabolic and transcription activities, present on large sized fibrillar center[29].

This study revealed that nicotine treatment resulted in disorganization of and dissociation of microtubules within the neuronal processes and blood capillaries. This would negatively affect axonal transport and may lead to neuronal death. In accordance with our results, microtubule disorganization was also reported in human lung epithelial cells of cigarette smokers, in the form of disturbed cellular morphology and viability in those cells[30]. The changes that occurred to blood capillaries in this study as collapse or contraction could be linked to the damage of microtubules. It can result from a defect in the oxidation of the tubulin system and was proposed to be related to the damage to the endothelium of blood vessels by the smoke extract[31].

In this study nicotine treatment resulted in disruption of structure of the myelin that surrounds neuronal processes. Smoking was previously associated with about 50% more risk of the occurrence of multiple sclerosis in case control studies[23, 30]. A proposed mechanism for initiating the development of MS is leakage of the blood brain barrier (BBB). This suggestion is supported by a study, which reported that continuous nicotine administration leads to defects in BBB permeability through modulation of tight junction proteins[33, 34].

Additionally the dilatation of astrocytic processes could be due to the increase in the thickness of the basement membrane. This is consistent with a previous work, which reported that following nicotine administration astrocytes have an enlarged watery cytoplasm with multiple vacuoles [9]. The increased thickness in the basement membrane of the vascular endothelium, detected in this work, is in line with a study, which reported increased expression of collagen type IV in BM of neonatal Balb/C mice brain due to maternal nicotine exposure[37].

In the current study the nicotine treated auditory cortex showed dark aggregations of fibrillary materials which were demonstrated with silver stains in which the stain was deposited on cytoskeletal components and appeared as black metallic silver aggregates. Similar results were also, observed by other investigators who described such aggregations as plaques or spherical areas with argentophilic fibrils in dystrophic neuritis[38]. This result can be compared to the neurofibrillary tangles of the Alzheimer’s disease, which are considered insoluble twisted fibers located in brain cells and grouped as neurofibrillary tangles which results in the disintegration of the transport system of neurons in brain cells[39].

To sum up, the administration of nicotine to adolescent guinea pigs resulted in marked degenerative changes to the auditory cortex, which was, manifested in their morphology and morphometry. This is stressing the deleterious effects of cigarette smoking in the auditory cortex and will affect auditory functions of the individuals particularly the adolescent young age, which is a vulnerable time for auditory and overall nervous system development.

CONFLICTS OF INTEREST

There are no conflicts of interest

REFERENCES


EFFECTS OF NICOTINE ON AUDITORY CORTEX OF ADOLESCENT GUINEA PIGS

T. Al Haddad Al Mundhir, A. Al Asfour, N. Al Hashmi, H. Al Suwaidi, and A. Al Khamees

Materials and Methods

The results showed significant changes in the auditory cortex of adolescent guinea pigs exposed to nicotine. The histological examination revealed structural alterations, including neuronal loss and gliosis. The statistical analysis confirmed the presence of nicotine-induced damage in the auditory cortex.

Conclusions

Nicotine exposure during adolescence affects the auditory cortex, indicating the importance of early intervention in smoking prevention.