

Full Length Research Paper

Smoke and ethanolic extract of *nicotiana tabacum* altered hippocampal histology and behaviour in mice

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The effects of tobacco use on human health are well known and are documented in scientific reports. When tobacco is smoked or chewed, nicotine is absorbed by the lungs and oral cavity and quickly moved into the bloodstream where it is distributed rapidly through the circulatory system to reach the brain and peripheral nervous system. Male and female adult mice (N=32) were used for this study. The animals were randomly divided into four (4) groups, A, B, C and D, of eight (n=8) animals each. Group A animals were treated by oral administration of 10.72 mg of the extract, B 10.72 mg of the tobacco smoke exposure for 3 min (in a controlled air chamber), C were given 0.2 ml of normal saline and D were exposed to equal weight (0.02 g) of cotton wool for 3 min, for 21 experimental days. The mice were sacrificed 4 h after the last administration and the brains excised, blotted, weighed and fixed in formol calcium for histological analysis, using Haematoxylin and Eosin. There was a significant decrease in the body weight, brain weight and relative brain weight in the treatment groups. The pyramidal and granular cell layers showed changes in cell count scores; significant at $p < 0.05$ when compared with the control. The results suggested that the consumption of *Nicotiana tabacum* leaves; either smoking or chewing may lead to alterations in cell count, brain weight and neurobehavioral patterns. Weight loss was also observed in the treatment and was found to be dependent on the route of administration.

Key words: Nicotine tobacco, hippocampus, memory, cell death.

INTRODUCTION

The role of the cholinergic systems has been established over the years in neuropsychiatry research. The cholinergic system constitutes part of the reward feedback mechanism of the brain and nervous system (Janes et al., 2014). This is characterized by the presence of acetylcholine at the synapses (Wu et al., 2014). The cholinergic neurons are diffused in the brain and are excitatory in nature (Shinohara et al., 2014). This

is also found at the neuromuscular junctions where it facilitates muscular contraction and mechanical activity in the body (Gallego et al., 2013). Other sub-classifications of the cholinergic receptors involves the nicotinic and muscarinic receptors located in glands, the cells of the gastro intestinal tract and the lungs (Chan et al., 2013).

The nicotinic receptors are the sites of activity of the nicotine and are widely distributed in the CNS and

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neuromuscular junctions (Chan et al., 2013; Zhu et al., 2013). Cellular mechanism studies reveals that potentiation of nicotinic receptor by nicotine is in a manner similar to that of acetylcholine. Thus, nicotine is a cholinergic agonist (Sharma et al., 2014). Other structural analogues of nicotine and acetyl choline will potentiate the nicotinic cholinergic receptor (Cahill et al., 2014; Gannon et al., 2013).

Addictive properties of nicotine are linked directly with the positive reward system of the brain. This is because, nicotine can provide prolonged stimulation of the cholinergic receptors, keeping the brain and neuromuscular junctions active. Other behavioral changes includes profuse sweating, dilation of the pupil, restlessness and hyper activity (Smith and Chapman, 2014; Zwart et al., 2014). At the onset, efficiency is recorded in the activity of such individuals. In prolonged use (addiction), over stimulation of nicotinic cholinergic receptors leads to excitotoxicity and neuronal cell loss characterized by disorientations and other neuropsychiatric symptoms (Umene-Nakano et al., 2014).

The prolonged activity of nicotine in the synapses has been reported. The neuronal metabolic machinery is endowed with systems that are capable of regulating and reducing cholinergic stimulation of acetylcholine (Jain et al., 2013). Acetyl cholinesterase (AChE) often deactivates the cholinergic stimulation and breaking down acetyl choline into choline and acetate both at the synapse and neuromuscular junction. However, AChE is incapable of breaking down nicotine, thus a prolonged excitation at the cholinergic sites (Hashimoto et al., 2013).

Hippocampus is the structure that lies on the fringes of the medial aspect of each cerebral hemisphere (the limbic system) of the brain (Yin et al., 2014). Exposure to tobacco (nicotine) either from cigarettes and other forms of tobacco including cigars, pipe tobacco, snuff and chewing tobacco has been reported to be associated with alteration in the normal functions of the brain and the whole nervous system (Stephen, 1999; Charles, 2000; Anthony, 2002; NIDA, 2009A). Nicotine has been reported to be the highest and most toxic compound of aqueous extract of tobacco leaves (Sas, 1990; Leroy, 1999; Philip, 2002). However, therapeutic uses of nicotine include its use to aid smoking cessation and other nicotine addictions (Charles, 2000; NIDA, 2009A). Using a controlled amount of nicotine helps to reduce nicotine withdrawal symptoms when one attempts to quit the use of tobacco products (Charles, 2000; Adeniyi, 2007; NIDA, 2009A). Annually, about 5 million deaths is attributed to tobacco smoking contributing the second leading cause of mortality among adults worldwide (Aghaji, 2008; Uwakwe and Modebe, 2008). This frightening data attests to the death of about three million people in the year 2007 alone (WHO, Resolution, 1993; World Health Statistics, 2007), these findings and reports suggest the need for further experimental and clinical studies of the role of tobacco intake on the body systems,

most especially the brain in particular and the aim of this study is to investigate the effects of both ethanolic and smoke extract of tobacco on the hippocampus of juvenile mice.

MATERIALS AND METHODS

Animal care

All experimental investigations were done in compliance with humane animal care standard outlined in the "Guide to the Care and Use of Animals in Research and Teaching", as approved by the Institute of Laboratory Animal Resource, National Research Council, DHHS, Pub. No NIH 86 - 23 (1993).

The study was carried out using healthy juvenile mice of both sexes (18 - 25 g). The animals were kept under standard laboratory conditions (12 h light and 12 h darkness, temperature, humidity and ventilation). They were given standard rat diet, purchased from the same company, Bethel Feeds, Ilorin, Nigeria.

Extract preparation

The *Nicotiana tabacum* leaves pack was collected from Igboho, the northern part of Oyo State, Nigeria. Plant samples were authenticated at the Department of Plant Science, University of Ilorin, Nigeria. The leaves were air-dried at room temperature. 50 g of the blended leaves was dissolved in 500 ml of 70% alcohol for 24 h at room temperature. The filtrate was thereafter obtained from the solution using Whatman's No 1 filter paper and evaporated to dryness in an air-dry oven at 40°C. The residue of the extract obtained in form of paste was stored in a capped bottle and kept in a desiccator (Carla et al., 1997; Adeniyi et al., 2010). The pH of the extract was determined to be 4.19 before concentration and 5.72 after concentration. This was done using a pH meter (pHs - 25 Model). The yielding rate of the tobacco extract was determined to be 41.35%.

Animal treatment

The animals were given the *N. tabacum* as shown in the Table 1.

The tobacco extract

This was given orally with the aid of an orogastric tube.

The tobacco smoke

This was administered by exposing the animals to dried *N. tabacum* leaves wrapped with 0.02 g of cotton wool in a burning chamber for three minutes [Burning time (BT); this was determined by allowing three of the *N. tabacum* leaves of known weight (10.72 mg) to burn and their average burning time was determined]. The administration was done for 21 days and 4 h after which mice from each group was sacrificed for analysis; while the rest were sacrificed by 7 days (a week) after the last administration, to study the withdrawal effects of the *N. tabacum* exposure on the animals.

Experimental design

Total of N=32 mice (n=16 males and n=16 females) were used for this study. The animal was randomly divided in to four (4) groups, A, B, C and D, of eight (n=8) animals each. Group A received 10.72

Table 1. Brain weight (g) and relative brain weight (RBW) changes in animals during the experimental period (mean \pm SEM).

Group	Day 21		Day 28	
	BWT	RBW (%)	BWT	RBW (%)
A	0.5172* \pm 0.0112	2.57	0.3786* \pm 0.0209	1.89
B	0.4121 \pm 0.0121	1.93	0.3667 \pm 0.0072	1.66
C	0.3434 \pm 0.0122	1.61	0.3080 \pm 0.0066	1.28
D	0.03623 \pm 0.0212	1.52	0.3480 \pm 0.0370	1.42

*Significantly different from control mice ($P < 0.05$).

mg of orally administered extract, B 10.72 mg of the tobacco smoke exposure for 3 min. The animals in Group C were given 0.2 ml of normal saline, while the treatment Group D was exposed to equal weight (0.02 g) of cotton wool for 3 min, for 21 days.

Neurobehavioural observations

The neurobehavioural analysis was done at 0800 h of the day using Elevated Plus Maze (EPM) to study the locomotion, exploration and motor coordination in both the treated and control animals. The results are shown in Figure 3.

Animal sacrifice

After administration, the mice (four from each group) were sacrificed by cervical dislocation at days 21 and 28 of the treatment and their brains were excised, blotted with filter paper and the wet weights were taken and recorded, and brains were quickly transferred to a specimen bottle containing 10% formal calcium and fixed for two days (Bancroft and Stevens, 1990). Thereafter, the hippocampus was excised to process for histological analysis and the wet weights of the brain and volume was recorded for analysis. The brain volume was determined by liquid (water) displacement method and recorded in millimeter cube (mm^3).

Histology

The brains are fixed in 10% formal calcium, hippocampus were excised and processed for Haematoxylin and Eosin (H&E) and Cresyl Fast Violet (CFV) staining technique (Bancroft and Stevens, 1990). The slices of 5 μ were sectioned with the Letiz rotary microtome. The sections were mounted and examined with the light microscope. Image acquisition was done using the Cameroscope 5.5 connected to a computer interface and mounted on the Olympus binocular research microscope.

Morphometry

The pyramidal (PCL) and granular (GCL) cell layers thickness were measured using the method of W.H.O (1991) and Ofusori et al. (2008) in which an oculometer (10X10 grid lines) was inserted into the microscope and focused through stained slides.

Statistical analysis

The data were expressed as means \pm standard error of mean

(SEM). The data were statistically evaluated with SPSS software version 14.0 software. One-way analysis of variance (ANOVA) at significant level $2\alpha = 0.05$ were used.

RESULTS

Physical examination

There were no significant changes in the skin colour and arrangement; the colour of their eyes was normal as compared to the control groups. Also, the gross anatomy of the brain of the experimental appeared normal as compared to the control groups.

Weight changes

The average weight gain recorded for treatment group during the experimental period was reduced during the first 14 days in group A and B as compared to C and D. However, all the treatment groups recorded an increase in average weight during the 7 withdrawal days.

Neurobehavioral parameters

The general behaviour of the animals was comparatively normal. However, rate of head deeding (HD), stretching (S), quadrature duration (QD) and transition (T) were significantly ($p < 0.05$) different between experimental groups and the control groups (Figures 1 to 4).

Brain weight (BWT) changes

The average brain weight recorded for treatment group during the experimental period reduced during the 7 days of withdrawal (Table 1).

Relative brain weight (RBW) changes

The RBW changed between the experimental groups; those in group A had the highest RBW as compared to C and as in group B as compared to D after 21 days of

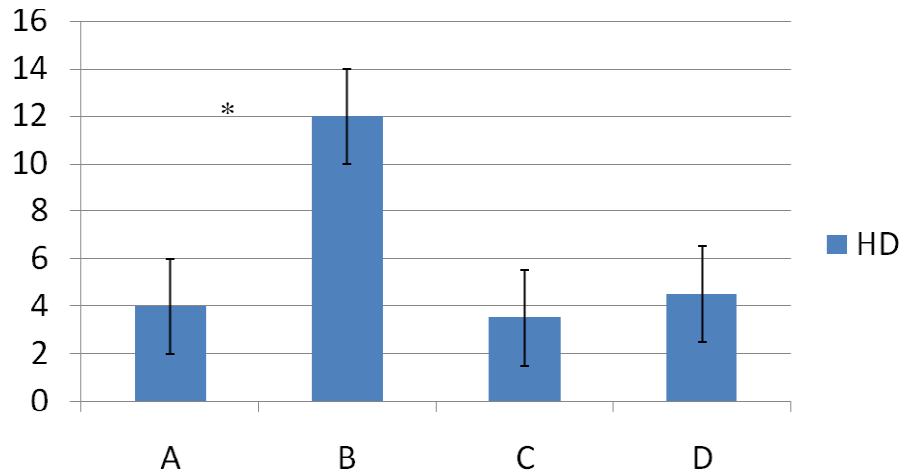


Figure 1. Head dipping (HD) of mice after 21 days of *Nicotiana tabacum* exposure.

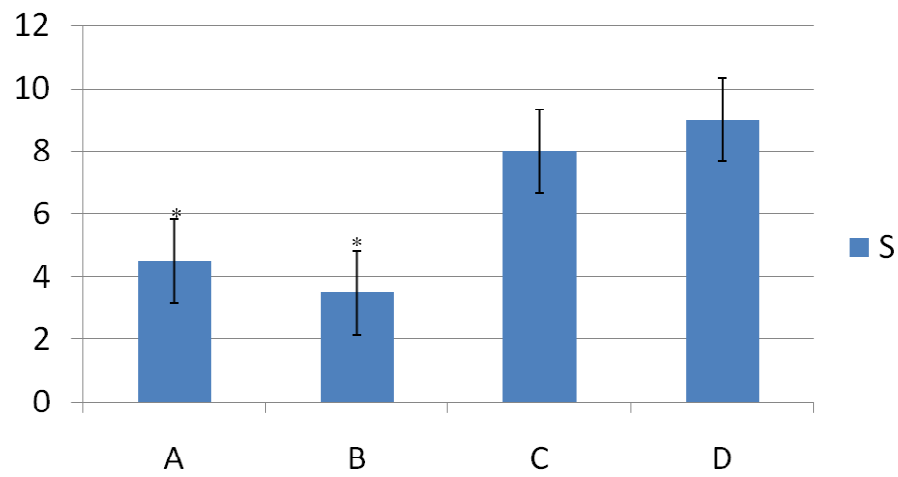


Figure 2. Stretching attempt (S) of mice after 21 days of *Nicotiana tabacum* exposure.

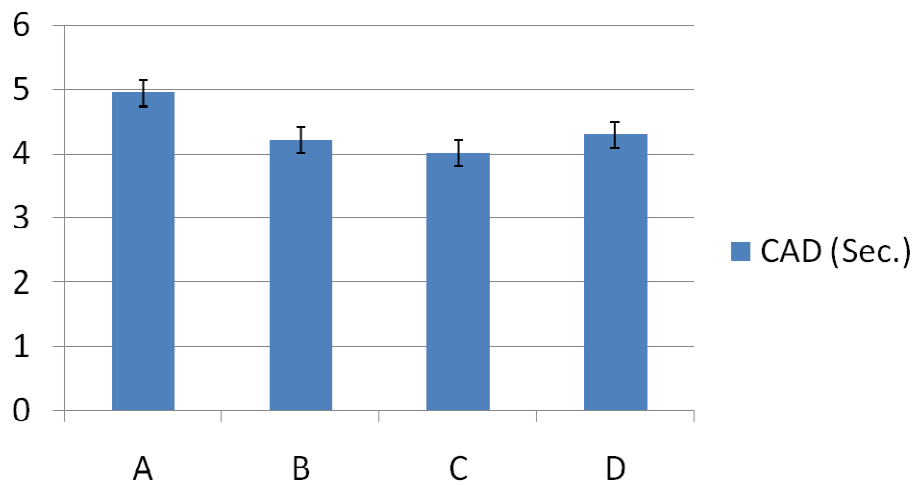


Figure 3. Close arm duration (CAD) in seconds of mice after 21 days of *Nicotiana tabacum* exposure.

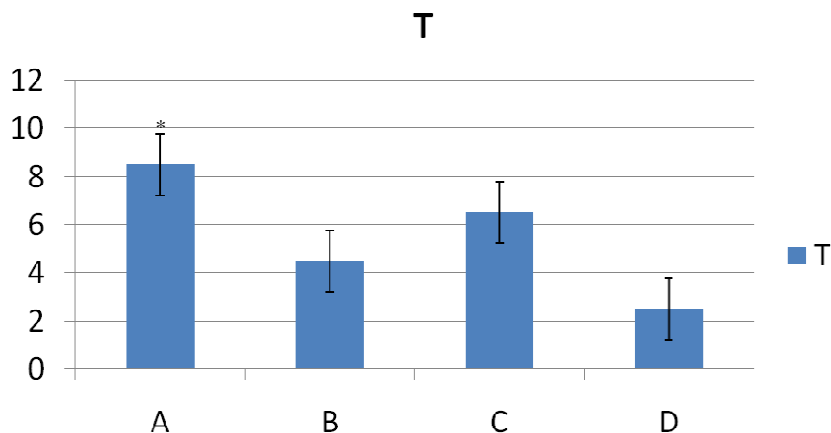


Figure 4. Transition (T) of mice after 21 days of *Nicotiana tabacum* exposure.

Table 2. Brain volume (ml) changes in animals during the experimental period (mean \pm SEM).

Group	Day 21	Day 28	Percentage brain volume changes (%)
A	4.0 \pm 0.00	3.5 \pm 0.50*	(12.50)
B	4.0 \pm 0.00	4.0 \pm 0.00	0.00
C	4.0 \pm 0.00	4.0 \pm 0.00	0.00
D	4.0 \pm 0.00	4.0 \pm 0.00	0.00

*Significantly different from control mice (P<0.05).

treatment and this is dose dependent (Table 1).

Brain volume (BRV) changes

The volume of brain of the animals was relatively the same in both experimental and control groups. Although, there was slight (12.5%) increase in the brain volume in group A, there brain volume changes were relatively the same across the groups (Table 2).

Hippocampal neurohistology (Plate 1)

Cell body stain intensity

The cell bodies are more densely stained in the experimental groups in a dose dependent manner as compared to the control groups and the architectural arrangement appeared normal.

Vacuations

There are more vacuulations in the experimental groups as compared to the control group C.

Cell population

The population of the neural cells (pyramidal cells) appeared to be more in the experimental groups as compared to the control groups in dose dependent pattern.

Pyramidal cell layer

This appeared uniformly normal with cell bodies of experimental group densely stained as compared to the control group (Table 3).

Granule cell layer

This appeared uniformly normal with cell bodies of experimental groups densely stained as compared to the control group (Table 3).

DISCUSSION

The observed reduction in weight gain of the animals in the experiment may implicate nicotine in tobacco plant

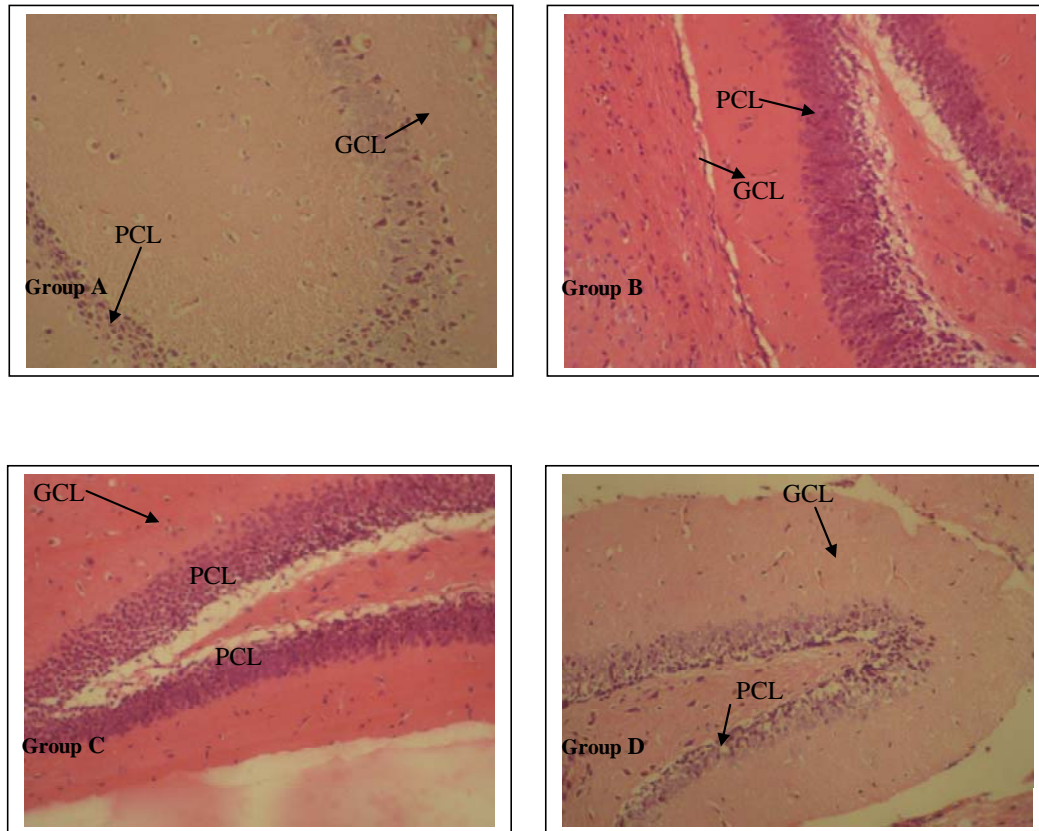


Plate 1. Hippocampus (H & E) at day 21: Magnification 480x. PCL: Pyramidal cell layer; GCL: granular cell layer.

Table 3. Hippocampal histometry analysis changes in animals at day 21 of tobacco exposure (mean \pm SEM).

Group	PCL ($\times 10^{-3}$ mm)	GCL ($\times 10^{-3}$ mm)
A	5.2000 \pm 0.6633	7.3333* \pm 1.4530
B	6.8000 \pm 0.9695	10.5000* \pm 1.3229
C	6.0000 \pm 0.4083	9.2500 \pm 1.1087
D	5.4000 \pm 0.4000	9.8000 \pm 1.2806

*Significantly different from control mice ($P < 0.05$).

use as reported by Chen et al. (2005) and this may be associated with reduction in food intake by the tobacco users. Also, the brain weight after administration and withdrawal were significant between the mice in group A as compared to those in groups C and D ($p < 0.05$), but those in group A have the highest relative brain weight (RBW) as compare to C ($p < 0.05$) and as in group B as compare to D ($p < 0.05$) after 21 days of treatment and this is dose dependent. This may account for the shift in the carbohydrate metabolic pathway due to stress induced by the activities of nicotine in the brain of the animals. The observed increase in locomotor activities in the mice (Figure 1 to 4) in the treated groups as compared to the

control groups reflect the possibility of tobacco administered to elevate anxiety characteristics in the treated groups which may probably explain the reason for increased cell density observed in the treated groups as compared the control groups (Adeniyi et al., 2010). Long term behavioral effects and molecular changes in brain mechanisms are resultant from nicotine exposure either through smoke or tobacco chewing. This is often evident and prominent in the adolescence humans (Romano et al., 2013). Depression is a characteristic long term behavior that may result from nicotine addiction (van der Meer et al., 2013). Long term smokers are more likely to suffer negative mood changes after nicotine withdrawals, thus contributing to their high morbidity and mortality from smoking related illness.

Several biochemical changes will often precede the structural changes observed in the hippocampus of the treated groups. The pattern of such changes might differ from the effect of nicotine exposure on the cortex owing to the effect of neurogenesis in the hippocampus. Our findings also suggests that nicotine administration, although was indifferent on gross brain structure, created a wide variation in behavior and memory. Considering the duration of our experiment, the effect of the treatment on long term memory cannot be elucidated. However, short

term memory processing is also associated with the hippocampus. The findings suggested that movement and cognition was reduced in the treatment group. This was also evident from the histological analysis with a reduction in cell count observed in the nicotine treatment group.

Comparative histology for the nicotine treatment groups A and B shows that tobacco smoke gave a more detrimental effect versus aqueous extract. To improve the model for studying nicotine dependence, we have employed the use of oral (aqueous) and smoke administered nicotine in tobacco.

Conclusion

Above all, from all the changes observed from comparisons between the experimental and control groups, it is safe to conclude that the administration of tobacco leaves smoke and extract can result in body and brain weight loss, distorted carbohydrate metabolism and alterations in locomotor activities.

Conflict of interests

The author(s) have not declared any conflict of interests.

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