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The Effect of Long Term Consumption of Powdered Tobacco Diet on Learning and Memory in Swiss White Mice

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Authors' contributions

The work was carried out in collaboration between all authors. Author BIO performed most of the experiments and wrote the first draft of the manuscript. Author SAB managed the analyses of the study and wrote the final draft of the manuscript. Author IEJ managed the literature searches and Author AAN performed the statistical analysis and Author EEO designed the study and wrote the protocol.

Original Research Article

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ABSTRACT

Aim: The effects of long term consumption of powdered tobacco (snuff) diet on learning and memory was studied in 30 Swiss white mice.
Design: The mice were randomly assigned into two groups (n = 15 each). Mice in group 1 (test group) were fed 1% tobacco diet while mice in the other group (control) were fed normal rodent chow only. Both groups were fed for 31 days before carrying out behavioural testing. All mice were given free access to clean drinking water.
Methodology: Food and water consumption by the animals were measured daily while their body weight was measured every two days. The Morris water maze was used to assess Learning and Memory in the mice.
Results: The mean daily water and food intake was significantly higher ($p<0.01$; 0.001

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respectively) in the tobacco diet-fed group than in control. The tobacco diet fed group also had a significantly higher ($p<0.05$) body weight gain than control. The swim latency during the reversal training was shorter in the tobacco diet-fed mice compared to their controls ($p<0.01$), showing better learning among the tobacco diet-fed group of mice. Southeast (reversal/retention) quadrant duration was also higher in the tobacco diet-fed mice ($p<0.01$) showing better memory than control.

Conclusion: Long term consumption of 1% tobacco diet increased food and water intake, weight gain and improved learning and Memory in mice.

Keywords: Powdered tobacco diet; food/water intake; learning and memory.

1. INTRODUCTION

Powdered tobacco otherwise known as snuff is widely used in Nigeria. It is prepared from the dry leaves of the plant, *Nicotiana tobacum*. Powdered tobacco is a type of smokeless tobacco. There are several types used in different ways but the most common is the dry nasal snuff, which is inhaled or "snuffed" through the nose [1]. Apart from tobacco leaves, other components are added to the powdered tobacco. The Nigerian snuff for instance is peculiar as it contains powered tobacco and some additives such as potash and notably, natron not found in snuff elsewhere [2]. Some people take it orally by simply chewing it in their mouth where it is externally absorbed via mucous membrane of the mouth into the blood stream. Although users of powdered tobacco believe it is safer than smoking, it still has many adverse effects. Apart from nicotine, tobacco leaves contain other alkaloids such as nornicotine, anabasine, myosmine and anatabine [3]. Among these constituents of tobacco, nicotine is the main constituent responsible for the majority of the psychological (e.g. dependence-forming) actions of tobacco [4]. Addiction to nicotine is one of the side effects of its use [5]. Another is cancer of the mouth and pharynx for which there are inconclusive reports [6,7]. Other side effects include; leukoplakia, gum recession, bone loss around the teeth, abrasion of teeth and bad breath [8].

Several studies on nicotine have found it to be a central nervous system (CNS) stimulant. Nicotine has been reported to be a stimulant or depressant to the brain depending on the dose [9]. In another research elsewhere, it was shown that nicotine increased the loco motor activity in rats [10]. Nicotine acts on the nicotinic acetylcholine receptors, specifically the ganglion type of nicotinic receptors present in adrenal medulla and elsewhere. Due to its affinity for nicotinic acetylcholine receptors (nAChRs), nicotine affects several brain functions [11], increasing or decreasing the activity of the central nervous system de pending the dose used and the duration of exposure. It also has effects on a variety of other neurotransmitters through less direct mechanisms.

The effect of Nigerian powdered tobacco which contains nicotine and other additives on learning and memory has not been investigated. A random questioning of snuffers shows that it makes them calm, concentrated and work better. Some say that it enhances their memory and learning, alertness and boldness. However, those who have quit the habit believe it gives them calmness, boldness but work output is not enhanced. Therefore, this study was aimed at investigating whether or not the powdered tobacco (snuff) affects learning and memory by using Swiss white mice as experimental animals.

2. MATERIALS AND METHODS

2.1 Preparation and Storage of Tobacco Diet

The locally prepared powdered tobacco (snuff) was purchased from a local producer at Uturu in Abia state, Nigeria and stored in an air tight container prior to use. One gram of snuff was mixed with 99g of ground rodent chow making a 1% tobacco diet weight per weight (w/w). The mixed feed was stored in a sac until required for use.

2.2 The Experimental Animals

Thirty Swiss white mice weighing between 18 – 25.5g were obtained from the animal house of Physiology Department, University of Nigeria and Enugu campus. They were fed with normal rat chow and given water freely and allowed one week to acclimatize before the experiments began. Animals were kept under standard laboratory conditions in well ventilated rooms, with temperature of about 25±2ºC, 12 hours of day light and 12 hours of darkness. The animal care was according to the "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) and national laws on the animal care. Appropriate approval was also obtained from the local ethical committees.

2.3 Preparation of Experimental Animals and Treatment

The mice were randomly separated into two groups, - control and test. Fifteen mice served as the test group of animals and were fed 1% tobacco diet. The other fifteen mice served as control and so were fed normal rodent chow alone. All the animals were allowed drinking water freely. This treatment was done for 31 days before the mice were tested for learning and memory in the Morris water maze. The animals were weighed every two days while their food and water intake was recorded daily.

2.4 Acute Toxicity Test: Determination of LD_{50}

To ensure that dose of powdered tobacco given to the test group of mice was safe; the acute toxicity of powdered tobacco (snuff) was estimated using Swiss white mice weighing between 24 and 25g. Graded percentages (%) of tobacco diet ranging from 0.5% to 10% tobacco diet (w/w) were fed to seven groups of mice comprising five mice per group. In another set of 7 groups of mice (n=6 per group), an ethanol extract of powdered tobacco (obtained by mixing 50g snuff in 200ml ethanol for 48 hours, filtering using What man No.1 filter paper and evaporating the filtrate at $50^{o}C$) was administered at doses 5000, 4000, 3000, 2000, 1000, 500 and 250mg/kg, intraperitoneally. The number of deaths in each group within 24-72hours was recorded. The LD_{50} was calculated using probit kill of the dose which is the formula or method proposed by Lorke [12].

2.5 Photochemistry of Powdered Tobacco

The photochemical analysis of powdered tobacco (snuff) was carried out using the guide to modern technique of plant analysis [13]. The photochemical constituents of the powdered tobacco (snuff) obtained from cured, dried and ground tobacco leaves are as shown in Table 1 below.

2.6 Experimental Protocol for Learning and Memory

The Morris Water Maze modified for mice was used [14]. The water maze was made of a circular plastic pool measuring 110cm in diameter and 20cm in depth. The pool was filled with water to a depth of 11cm. The water was left to sit overnight in order to attain room temperature. On each day the of the experiment, the water was made opaque by adding liquid milk to ensure camouflage of a white escape platform located in one of the quadrants. The platform was submerged by 1cm of the opaque water.

The pool was divided into four quadrants: Northwest, Northeast, Southwest and Southeast quadrants. Boundaries of these quadrants were marked on the edges of the pool with masking tape and labelled: North, South, East and West. A square solid block (10cm x 10cm) painted was used as the escape platform in the maze. The pool was located in a room measuring 3.6 x 3.4m. On the walls of the room were mounted several posters to act as visual cues. There was also furniture in the room that provided visual cues. During testing, the room was dimly lit with sun light passing through the window covered with thick and dark coloured curtains. Two experimenters remained in the same position throughout the experiment days observing and scoring the behaviour of the mouse in the water maze.

2.6.1 Procedure

Testing in the Morris water maze lasted for eight days. The first three days were acquisition training with an invisible platform. Days 4-6 were reversal training, again with an invisible platform but this time in the opposite quadrant. On the seventh day, a probe trial was conducted with no escape platform. On day eight, four trials were conducted using a visible platform.

During acquisition training, the hidden platform was placed in the centre of the northwest quadrant. Each animal received 4 trials of 60 seconds (maximum) per day. The starting positions of the animals were predetermined by a random chart, preventing any sequence of two trials to be repeated by the same animal during any other day. Possible start positions were at the boundaries of the quadrants (e.g. west, North, East or South). Each mouse was removed from its holding cage using a small clean 500ml plastic container to minimize handling stress. The animal was then placed into the water at the appropriate start position, facing the centre of the pool. The mouse was then permitted to explore the pool and to search for the hidden escape platform for 60 seconds. When the animal located the platform, the timer was stopped and the animal was removed using the plastic container and placed in a holding cage with paper towel. If the animal did not find the platform during the allotted time, the animal was guided onto the platform using the plastic container. Once on the platform, the mice were permitted to visually explore their surroundings for 10 seconds, at which point they were picked up in the plastic container and returned to the appropriate holding cage.

Reversal training began on day 4, with the hidden platform moved to the opposite quadrant (southeast quadrant). Each mouse again completed the same number of trials as in the acquisition training (4 trials per day) for another 3 days.

A probe trial was conducted on day 7. At this time, there was no escape platform at all in the maze. Each animal completed a single trial of 60 seconds. Each mouse was placed in the maze from one of the four possible start positions and allowed to explore the pool.

The visible platform task was conducted on day 8. The visible platform was placed in the Southwest quadrant of the pool and each mouse was given only 4 trials of 60 seconds (maximum) per trial.

The behaviour scored during acquisition and reversal training (days 1-3, 4-6) as well as the visible platform task (day 8) was the swim latency, defined as the time it took for the mice to locate the escape platform. During the probe trial, day 7, the following were scored:

1. Duration in each quadrant.
2. The number of times the mouse crossed the location of the platform during reversal training (Annulus reversal crossing).
3. The number of times the mouse crossed the location of the platform during acquisition training (Annulus acquisition crossing).

2.7 Statistical Analysis

Data collected during the study were expressed as mean ± standard error of mean (SEM). Values of $P<0.05$ were regarded as statistically significant. Statistical analysis was done with the aid of the computer software SPSS and Excel from Windows XP (Brain Series, China).

3. RESULTS

3.1 Photochemical Screening of Powdered Tobacco (Snuff)

Table 1 shows the result of the Photochemical screening of powdered tobacco (snuff) extracts using ethanol and water, with nicotine common in abundant quantities (+++) in both extracts. While the aqueous (water) extract contained, tannins, phlobatannins, saponins, flavanoids, steroids, terpenoids, cardiac glycosides, anthraquinones, polyphenol and reducing sugar in moderate quantities, terpenoids were more abundant (+++). The ethanolic extract contained fewer chemicals. There were no tannins, phlobatannins, saponins, terpenoids, anthraquinones and polyphenol. The ethanolic extract was however rich in steroid (+++) and cardiac glycosides (+++).

Table 1. Results of phytochemical screening of snuff

Chemical constituents	Ethanol extract	Aqueous extract
Nicotine	+++	+++
Tannins	-	++
Phlobatannins	-	++
Saponins	-	++
Flavanoids	+	++
Steroid	+++	++
Terpenoids	-	+++
Cardiac glycosides	+++	+
Anthraquinones	-	++
Polyphenol	-	+
Reducing sugar	+	+

Key: Not present; +: Present in small quantities; ++: Present in moderate quantities +++: Present in abundant quantities

3.2 LD_{50} of powdered tobacco

Out of the graded percentages of tobacco diet ranging from 0.5% up to 10% tobacco diet (w/w) fed to seven groups of mice (n= 5 mice per group), no deaths were recorded and so the 1% tobacco diet given to test animals was considered safe for the experiments.

However, when various doses of the snuff were administered intra-peritoneally to another set of mice, some mice died at very high doses. The LD_{50} for intra-peritoneally administered snuff was 7943.28mg/kg body weight (Table 2, Fig. 1).

Table 2. Lethality study of powdered tobacco showing dose, percentage mortality and probit kill following intra-peritoneal administration of powdered tobacco extract

N	Dose (mg/kg)	Log dose (mg/kg)	Mortality	% Mortality	Probits
5	0	0.0000	0	0	0
5	100	2.0000	0	0	0
5	200	2.3010	0	0	0
5	400	2.6021	0	0	0
5	800	2.9031	0	0	0
5	1600	3.2041	0	0	0
5	3200	8 3.5052	2	40	1.7467
5	6400	3.8062	4	80	5.8416

LD_{50} of snuff (7943.28mg/kg)

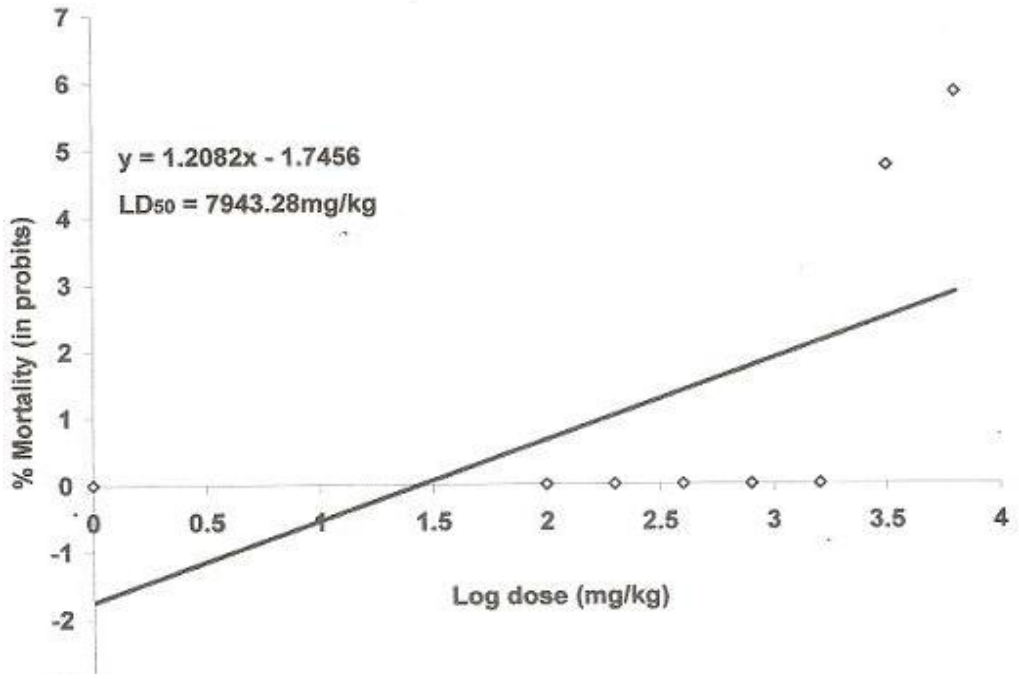


Fig. 1. Lethality studies showing the effects of administering graded doses (100 to 6400mg/kg I /p) of tobacco against the percentage mortalities (converted to probits)

3.3 Comparison of Daily Food Intake, Water Intake and Body Weight Changes between Powdered Tobacco Diet-fed Mice and Control

The daily food intake is shown in Fig. 2. The graph shows that the daily food consumption did not differ significantly until days 11 to 13 when it became higher in the tobacco diet-fed group of mice compared to control ($p<0.05$). Throughout the remaining days there did not seem to be any significant difference in the daily food intake. However, a comparison of the mean daily food intake (Fig. 3) showed that the tobacco diet-fed mice consumed more food than their controls ($p<0.01$).

The result of the water intake shows that the powdered tobacco diet-fed mice drank more water than their control from the first days 1 to 8 and from day 14 to day 25 of the study Fig. 4. The mean daily water intake Fig. 5 showed the water intake was significantly greater in the tobacco diet-fed group of mice than in control ($p<0.01$).

The changes in body weight are shown in Fig. 6. The powdered tobacco diet-fed group of mice showed greater weight gain ($p<0.05$) when compared with control at the end of day 31.

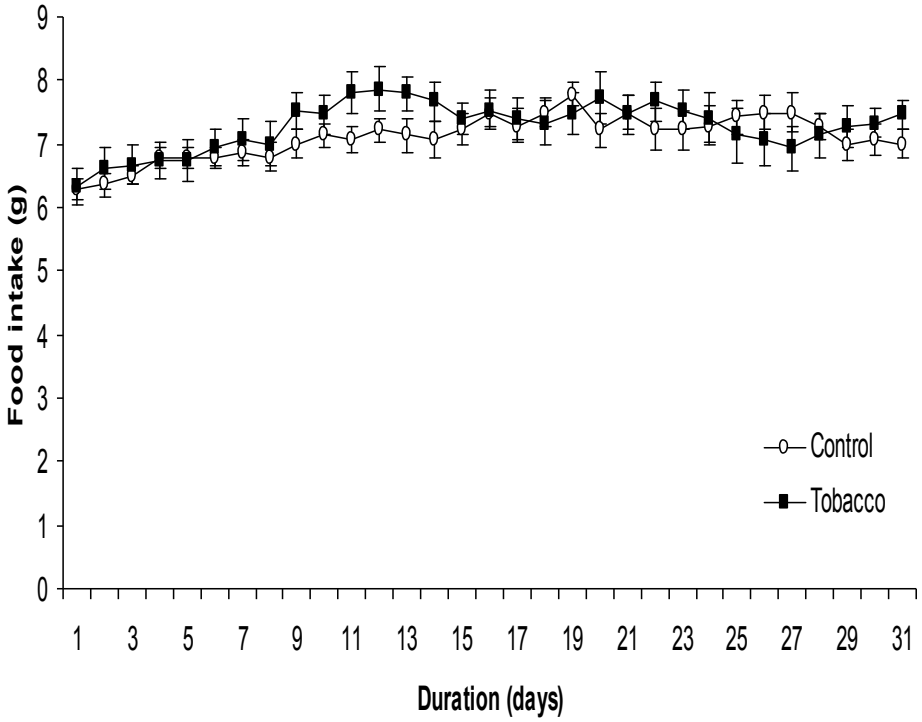
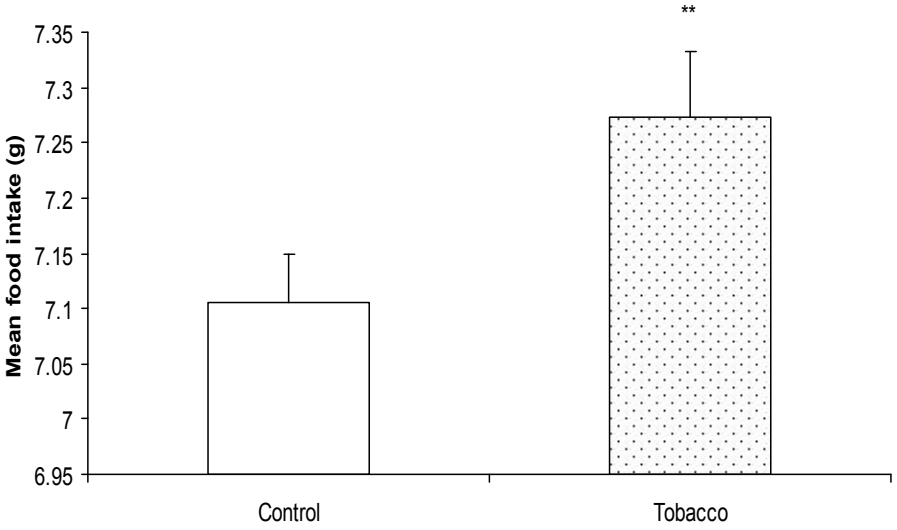


Fig. 2. Comparison of daily food intake between powdered tobacco diet-fed mice and control



*** - significant at p< 0.01 compared to control*

Fig. 3. Comparison of mean food intake between powdered-tobacco diet-fed mice and control

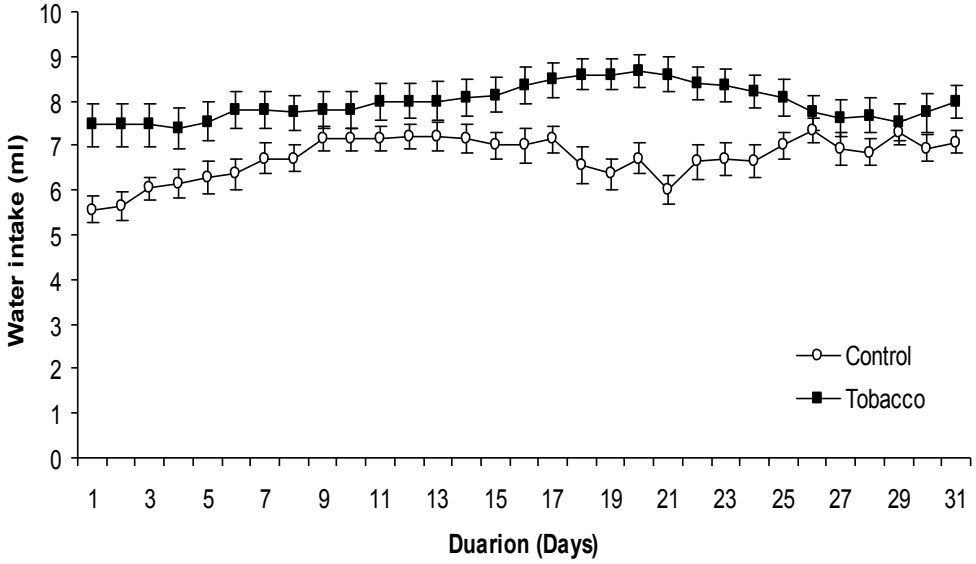
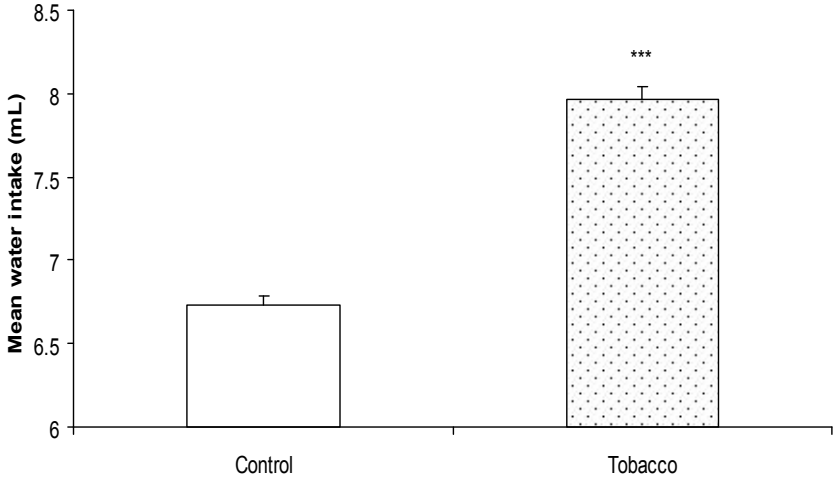
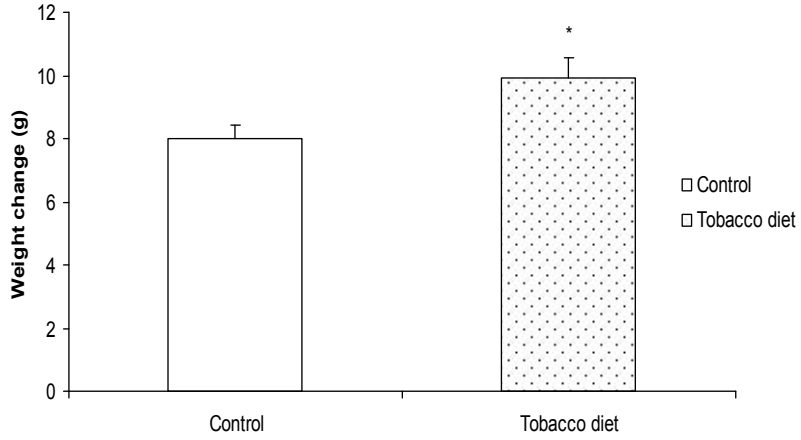


Fig. 4. Comparison of daily water intake between powdered tobacco diet-fed mice and control



*** - significant at p< 0.001 compared to control

Fig. 5. Comparison of mean water intake between powdered tobacco diet-fed mice and control



* - significant at p< 0.05 compared to control

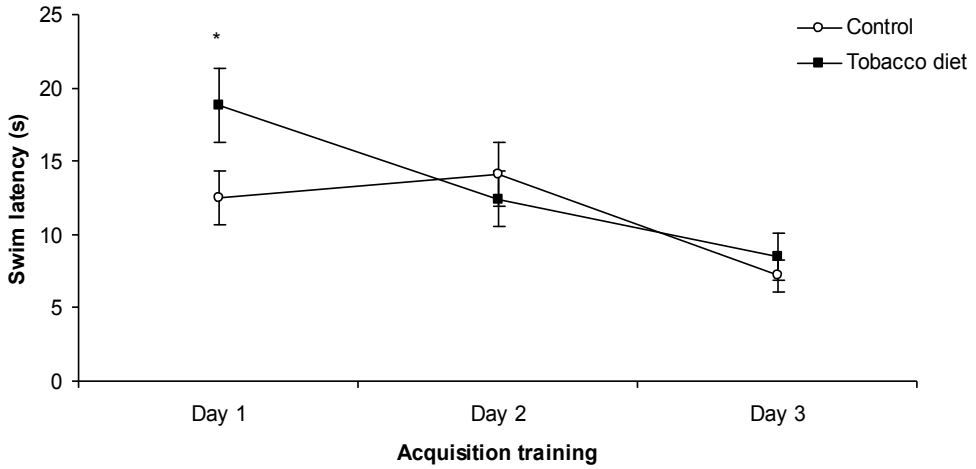
Fig. 6. Comparison of mean body weight change between tobacco diet-fed mice and control

3.4 Comparison of Results in the Morris Water-maze Test between Powdered Tobacco Diet-fed Mice and Control

The swim latencies scored during the 3-day acquisition training is shown in the learning curve on Fig. 7. The swim latency of the tobacco group of mice in acquisition day 1 was significantly longer than that of the control group ($p<0.05$). Those of days 2 and 3 were not significantly different.

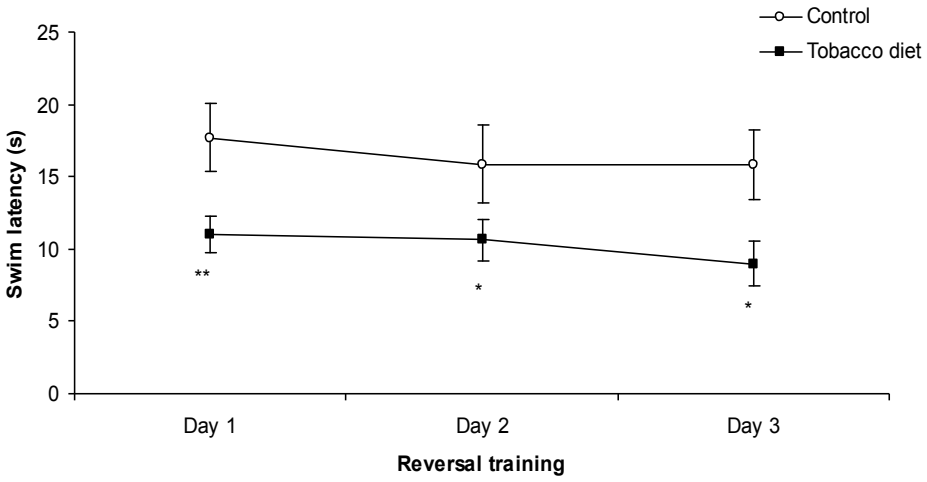
The swim latencies in the learning curve during reversal training (Fig. 8) showed significantly shorter swim latencies in the powdered tobacco diet-fed group of mice on day 1 ($P<0.01$), and on days 2 and 3 ($P<0.05$) compared to their control.

Fig. 9 compares the quadrant duration during the probe trial between the tobacco diet-fed mice and control. The results showed that the tobacco diet-fed mice spent more time exploring the Southeast quadrant (platform location during reversal training) compared to their control ($P<0.01$). The swim latencies during the visible platform test (Fig.10) were significantly shorter ($p<0.05$) in the tobacco diet-fed mice compared to control.



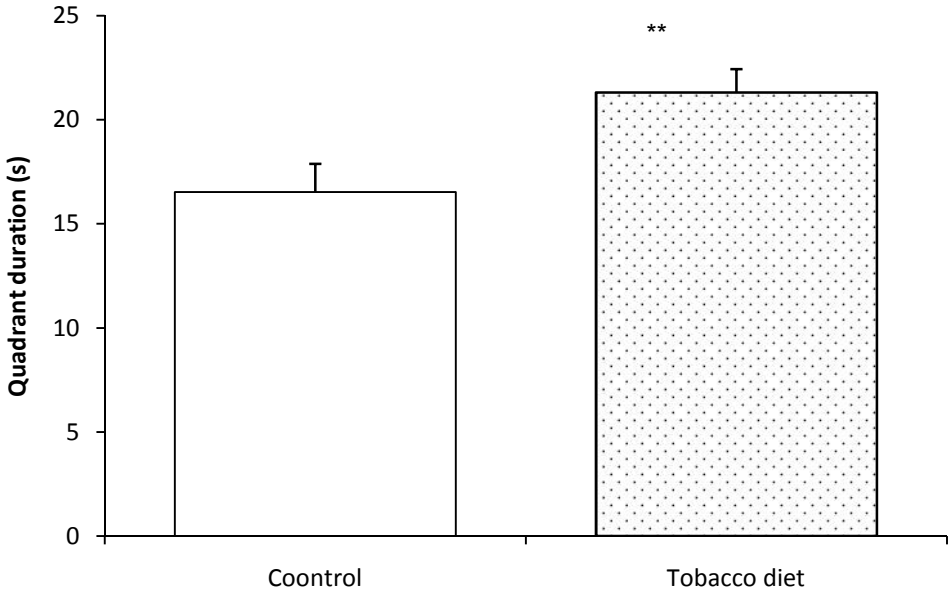
** - Significant at p< 0.05 compared to control*

Fig. 7. Comparison of swim latencies during the acquisition training in the Morris water-maze test following consumption of control diet and Tobacco diet in mice



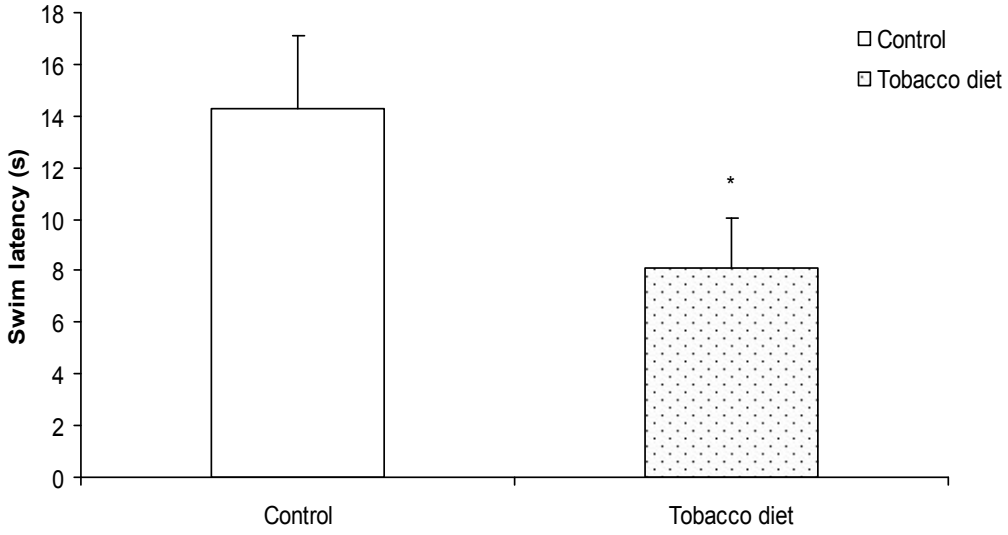
** - Significant at p< 0.05 compared to control. ** - Significant at p< 0.01 compared to control*

Fig. 8. Comparison of swim latencies during the reversal training in the Morris water-maze test following consumption of Tobacco diet and control diet in mice



*** - Significant at p< 0.01 compared to control*

Fig. 9. Comparison of Southeast (SE) quadrant duration during the probe trial in the Morris water-maze test following consumption of control diet and Tobacco diet in mice



** - Significant at p< 0.05 compared to control*

Fig. 10. Comparison of swim latencies during the visible platform task in the Morris water-maze test following consumption of normal and Tobacco diet in mice

4. DISCUSSION

The aim of this study was to investigate the effects of long term consumption of powdered tobacco (snuff) on learning and memory in Swiss white mice. However, the Phytochemistry and LD_{50} of powdered tobacco were also investigated to give information on the active constituents of the snuff and ascertain a safe dose for administration. The effects of powdered tobacco on water intake, food intake and body weight change were also studied.

4.1 Phytochemistry of Powdered Tobacco

From the results of the phytochemical analysis of powdered tobacco, bioactive natural compounds namely, nicotine, tannins, phlobatannins, saponins, flavonoids, steroids, terpenoids, cardiac glycosides, anthraquinones, polyphenol and reducing sugars were present. This phytochemistry revealed more biologically active constituents those earlier reports by Andersen and Kasperbauer [15], Leffingwell [16] and Maduka, et al [2]. Nicotine has however, been consistently common in all these reports as a major alkaloid and present in high quantity.

4.2 LD_{50} of Powdered Tobacco

Although the 0.5% to 10% tobacco diets, taken orally ad libitum, did not show any lethality, intra-peritoneal administration of tobacco extracts showed lethality. So, 1% tobacco diet was seen as very safe and non-toxic. This 1% of tobacco diet relatively corresponds to the amount taken as snuff. Following intra-peritoneal administration of tobacco extracts, deaths were recorded at very high doses. The LD_{50} for intra peritoneal administration of snuff was calculated from the probits to be 7943.28mg/kg. Our study was however, limited to oral administration as food.

4.3 Food Intake, Water Intake and Body Weight Change

The mean food intake for the period of the experiments showed that the tobacco-diet fed mice ate more than the control group. This means that tobacco intake increased food consumption. Similarly, water intake was also higher in the tobacco-diet fed group of mice. So, tobacco here also increased water consumption. A look at the results on body weight changes also showed increased weight gain in the mice following chronic consumption of the tobacco diet. This is expected since there was a significant increase in both food and water intake in this group of mice fed tobacco diet.

Our results are, however, very interesting because chronic administration of nicotine, the major addictive component of tobacco, reduces appetite and alters feeding patterns decreasing food and water intake, typically resulting in reduced body weight [17]. This is not only restricted to human subjects but also applies to experimental animals [18,19,20]. Particularly striking is the hyperphagia and resultant weight gain that accompanies smoking cessation of 70–80% of people who quit smoking [21,22].

In our study, we observed that long term consumption of tobacco diet caused increased food and water intake as well as body weight gain. The reason our result on tobacco consumption negates previous results on nicotine is not clear. This leaves room for further research. However, there is a possibility that since steroids increase protein synthesis, promote growth

of muscles and bones, increase food and water intake and ultimately increase body mass in man [23,24], the steroids present in the tobacco could have caused these effects.

4.4 Learning and Memory in the Water Intake Maze Task

Learning means the ability to add knowledge to the brain while memory is the ability to remember what was learnt [25]. The entire brain is involved in learning and memory. However, specialized learning requires special areas of the brain. Therefore, a substance that generally stimulates the central nervous system and increases alertness will improve learning and memory. The Morris water maze was used to study learning and memory. The hidden platform version of the Morris water maze tests for visuo-spatial learning and memory, which is hippocampus dependent [26]. The visible platform version of the Morris water maze is a non-hippocampal task, which is dependent on the caudate nucleus and put amen of the basal ganglia. The visible platform uses a unique intra-maze visual cue that is placed at the location of the escape platform whereas the visuo-spatial learning task uses extra-maze cues [26].

The behaviours scored included swim latencies during the acquisition, reversal training and visible platform task, and the quadrant duration during the probe trial. Shorter swim latencies indicate faster learning, as the mice are able to locate the hidden escape platform within a shorter time. This was observed in the tobacco diet-fed group of mice particularly during the reversal training period of the test, although it did not differ for most of the acquisition training.

During the probe trial, the time spent in the South east quadrant which is the location of the hidden platform during the reversal training, also called the retention quadrant, is a measure of how well the animals remembered the position of the hidden platform. The tobacco diet-fed mice spent more time exploring the retention quadrant when compared to their controls. This indicates that these mice had better visuo-spatial memory of the platform position, which is a hippocampus dependent function. In the visible platform task, the tobacco diet-fed group of mice had shorter swim latencies. Shorter latencies indicate better performance in the task. This also indicates better non-hippocampus learning in the tobacco diet-fed group compared to control and which is consistent with the other results.

Therefore, our studies showed that tobacco diet improved both hippocampus- and non-hippocampus-dependent learning and memory. This is very consistent with the findings that nicotine, the main addictive substance of tobacco, affects several brain functions due to its affinity for nicotinic acetylcholine receptors (nAChRs) [11]. It particularly affects learning and memory in some of the reports listed here. Nicotine improves long-term spatial memory, as measured in the Morris Water Maze [27] and procedural learning [28]. Administration of nicotine also enhances cognitive functions in pathological conditions such as Alzheimer's disease [29] and it reverses memory deficits caused by a lesion of the cholinergic system [30].

5. CONCLUSION

Nigerian powdered tobacco (snuff) stimulates water intake, food intake and body weight gain in mice, contrary to the reports of other researchers on studies with nicotine, one of the most neuro-active constituents of tobacco. Tobacco di*et al*so enhances both hippocampus and non-hippocampus dependent learning and memory in the mice. If these results are

applicable to man, consumption of 1% tobacco-diet could be beneficial in improving learning and memory in humans. However, the potential unexpected effects of tobacco consumption must be kept in mind.

CONSENT

Not applicable.

ETHICAL APPROVAL

The authors herein declare that the “Principles of laboratory animal care” (NIH publication No. 85-23, revised 1985) as well as national laws on the care of animals were strictly adhered to during the experiments. Appropriate approval was also obtained from the local ethical committees.

COMPETING INTEREST

The authors hereby declare that there was no conflict of interest.

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