Effect of Smokeless Tobacco on the Hippocampus of Adult Male Wistar Rat

OBOT JOSEPH IME[1], OKOSEIMIEMA SONNY CLEMENT[2]
[1, 2]*Department of Human Anatomy, University of Port Harcourt*

Abstract- ***The use of smokeless tobacco products is common worldwide, with increasing consumption in many countries. The various forms of smokeless tobacco used depends on culture, social, and geographical variation. Although smokeless tobacco use causes adverse health outcomes, understanding its use and the impact is made difficult by the wide variation in products and user-specific behaviours. This diversity makes generalising about these products across regions and countries as one entity inaccurate. This research evaluated the effects of snuff consumption on the CA3 region of the hippocampus of adult male Wistar rats. 24 adult male Wistar rats were randomly assigned into four groups: group I, II, III and IV. Group I received only distilled water, group II received 20 mg/kg, group III received 30 mg/kg and group IV received 40 mg/kg of the aqueous extract of smokeless tobacco orally through oral gavage. The duration of the administration lasted for 33 days. The rats were sacrificed on day 3 and day 33 to extract the brain tissue for histological processing. Histomorphological examination revealed progressive histopathological changes which includes pkynosis, chromatolysis, vacuolation and gliosis. These changes were mild in day 3 but increased extensively in day 33. The result from group III and IV administration suggests progressive neurotoxicity which was time and dose-dependent. In conclusion, this study demonstrated that exposure to aqueous extract of smokeless tobacco produces progressive neurodegeneration in the hippocampus of adult male Wistar rat.***

I. INTRODUCTION

Snuff, a smokeless tobacco product, has been used for centuries and is prevalent in various cultures worldwide. Smokeless tobacco may refer to various substances like tobacco snuff, dipping, snus, tobacco gum, tobacco toothpaste, tobacco paste, herbal smokeless tobacco, tobacco water etc (Jena *et al*, 2016). It is estimated that more than 300 million people use it globally (Chugh *et al.,* 2023). It is consumed in unburnt forms through sniffing and chewing which contain several carcinogenic compounds (Jena *et al*, 2016). Nasal inhalation of fine, dry tobacco powder is an old practice. It was quite common and probably the predominant form of tobacco use in the 19th century (Sapundzhiev & Werner, 2003). With the advent of manufactured cigarettes, other forms of tobacco rapidly declined in the 20th century. According to National Cancer Institute, snuff tobacco is a type of smokeless tobacco that is made of finely ground or shredded tobacco leaves. It may have different scents and flavours and may be moist or dry. Moist snuff tobacco according to the institute is placed in the mouth, usually between the cheek and gum or behind the upper or lower lip. Dry snuff tobacco on the other hand is inhaled through the nose. The association between tobacco use and the risk of development of several health effects are well documented in literature. These consequences are borne by millions globally, contributing to morbidity and mortality for current and future generations (Aniwada *et al.,* 2018; WHO, 2019), which account for about 8 million deaths globally (Institute for Health Metrics and Evaluation (2024). This means that every 6 seconds, one person dies due to tobacco-related disease. About 75% of this mortality is among low- and middle-income country residents (Aniwada et al., 2018). With such staggering statistics about the effects of tobacco, extensive research has been conducted on the health consequences of smoking cigarettes. The negative health consequences and most of its effects are attributed to the principal constituent – nicotine (Ugwu & Aprioku, (2016). In Nigeria, the most popular tobacco smoke and smokeless tobacco products consumed are cigarette and nasal snuff respectively (Ugwu & Aprioku, 2016). Cigarette smoking does not only cause cancer, but has also been associated with increased incidence of respiratory tract diseases (Rabe, et al, 2007), coronary heart disease

(Jha, et al., 2008) and reproductive toxicity (Jauniaux & Burton, 2007, Aprioku & Ugwu, 2015). Apart from nicotine, tobacco products contain other components that are potentially toxic, carcinogenic, mutagenic, growth retardative and immunosuppressive. Such compounds include polycyclic aromatic hydrocarbons like benzopyrene, cyanide, carbon-monoxide, lead, cadmium, nitric oxide, nitric dioxide; tobacco-specific N-nitrosamine (TSNA), N-nitrosonornicotine (NNN), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), ammonia and flavours (Ugwu & Aprioku, 2016). The level of exposure or absorption of these substances from tobacco products depends among other factors on the type of tobacco used, this being thought to be higher in smokeless tobacco users than cigarette smokers (Desalu, et al., 2010; Brunnemann & Hoffmann, 1992).

Smokeless tobacco use adversely impacts health, with outcomes such as periodontal disease, mouth lesions and leukoplakia (Kabwama, Kadobera & Ndyanabangi, 2018). Also, a 2012 International Agency for Research on Cancer (IARC) review of multiple studies showed that smokeless tobacco causes oral cancer, esophageal cancer, pancreatic cancer in humans (Onoh et al., 2021). It is widely accepted that nicotine is the primary addictive constituent of tobacco, and there is growing body of evidence that nicotine demonstrates the properties of a drug of abuse (Balfour, 2004). Behavioural experiments with laboratory animals indicate that nicotine has psychostimulant properties similar to those of amphetamine and cocaine (Balfour, 2004). Studies of nicotine self-administration in various species, including humans, indicate that nicotine can serve as an effective positive reinforce (i.e., is rewarding), although in a more restricted range of conditions than for some other positively reinforcing substances such as cocaine (Henningfield & Fant, 1999). On neurocognitive effects, Loveness, Nabuzoka & Paul (2020) reported that the more one uses snuff, the more impairment in memory and attention. Recent studies have suggested that long-term use of smokeless tobacco could predispose to free radical generation and oxidative stress (Constance, Lusher & Murray, 2019; Avti et al., 2005). Previous studies have shown that low doses of nicotine can improve memory function and reduce plaque burden and could be used as an anti-Alzheimer disease agent and improve attention performance in schizophrenic patients (Barr et al., 2008; Swan & Lessov-Schlagger, 2007). Yusuf et al. (2021) reported that oxidative stress was induced by high-dose administration of smokeless tobacco and inhibition of acetylcholinesterase activity in male Wistar rats. These inconsistencies could be attributed to the doses. It appears high dosage of nicotine might induce neurotoxicity (Gupta et al., 2020) and excite oxidative stress, while a low amount could improve cognitive performance (Swan & Lessov-Schlagger, 2007). However, there is paucity of literature that has evaluated the histology architecture of the CA3 region of the hippocampus after consumption of smokeless tobacco, hence, this research was done to determine whether there are any alterations in the CA3 region of the hippocampus of adult male Wistar rats.

II. MATERIALS AND METHODS

Plant Collection and Identification

Grounded tobacco (snuff) was obtained from the Mile 3 market and identified by the Department of Plant Science and Biotechnology in the Faculty of Agriculture, University of Port Harcourt.

Extracts Preparation

The grounded snuff was sun-dried for 30 minutes. 40 grams of it was dissolved in 400 ml of distilled water and properly stirred. The mixture was allowed to stand in a clean corked container for 48 hours. Thereafter, it was filtered with a clean handkerchief and the filtrate was further filtered with Whatman 100 filter paper. The final filtrate was evaporated using a water bath at 60 °C. The dried extract was then stored in the refrigerator at a temperature of 4 °C till when dosing was done.

Ethical Clearance

Ethical clearance was sought and obtained from the Research and Ethics Committee of the University of Port Harcourt, Port Harcourt, Rivers State with the approval number UPH/CEREMAD/REC/MM102/024

Animals:

Twenty four (24) male Wistar rats were used for the study. They were kept in the Animal House of the Department of Human Anatomy, University of Port Harcourt. They were housed in a standard rubber cage

maintained in a well-ventilated room with 12 hours of light/dark cycle at room temperature. They were fed with standard rat chow and allowed access to tap water ad libitum. They were allowed to grow to maturity of 2 months where they clocked an average weight of 160 – 180 g before administration of extracts was done.

Grouping and Dosing

The adult Wistar rats were divided into 4 groups (6 rats in each group) labelled Group I, II, III and IV. Dried nasal snuff extract was dissolved in distilled water and given by oral gavage. Group I was given distilled water (control), group II was given 20 mg/kg b.w., group III was given 30 mg/kg b.w. and group IV was given 40 mg/kg b.w. The administration was done for 33 days.

The dosing was done following the guidelines given by Adias et al (2014) and Earnest & Ajaghaku (2014).

Tissue Extraction and Processing

The rats were first perfused with a fixative before the tissue was extracted. Tissue extraction was done in two time periods (day 3 and day 33). The process was the same in each of these periods. The perfusion pump/set-up was put in place first and test run with distilled water. The perfusion pump was replaced with 10% buffered formalin. The rats to be used for each of these periods were first anaesthetized using diethyl ether. Cotton wool soaked with diethyl ether was placed in a desiccator and the rat to be anaesthetised was introduced into the desiccator. Once the rat was unconscious, it was removed and placed on the operating table with its back down. The appendages were clamped unto the operating board using a thumb pin to ensure that the rat was securely fixed. Using a pair of scissors the fur on the abdomen was removed to expose the skin of the rat. With a scalpel, an incision was made through the abdomen. With a pair of sharp scissors, a cut through the connective tissue at the bottom of the diaphragm was made to allow access to the rib cage. With a pair of blunt scissors, the ribs were cut through the midline to gain access to the thoracic cavity. The open thoracic cavity was clamped to expose the heart. The beating heart was held steady using a pair of forceps, and then the perfusion needle was inserted through the left ventricle to extend to the aorta. The valve of the perfusion pump was then released to allow the fixative to flow. The atrium is then cut with a sharp pair of scissors. After about 20 minutes, the body of the rat was stiffened and the fixatives were seen dripping from the rat's nostrils and mouth. At about 40 minutes, when the rat has been properly fixed, it was observed that the rat was hardened, the liver appeared lightened, the perfusion was stopped and the head of the rat was excised. The flesh covering the skull was gently removed using a pair of scissors and the skull was placed in a specimen bottle filled with the 10% buffered formalin. After 3 days, the skull was cracked and the brain tissue was excised for tissue processing.

Tissue Processing (Histology)

The excised brain tissue was post-fixed in 10% buffered formalin for 24 hours. This fixative sufficiently filled the specimen bottle which the tissue was placed. The tissue was placed in the bottle such that it was freely suspended in the fixative and was not making contact with the bottom of the container. The brain tissues were later grossed picking out the hippocampus. The grossed tissue was placed in suitably labelled cassettes. The tissue was processed for photomicrography.

III. RESULT

The result of the cytoarchitecture of the hippocampus of the group I – General control

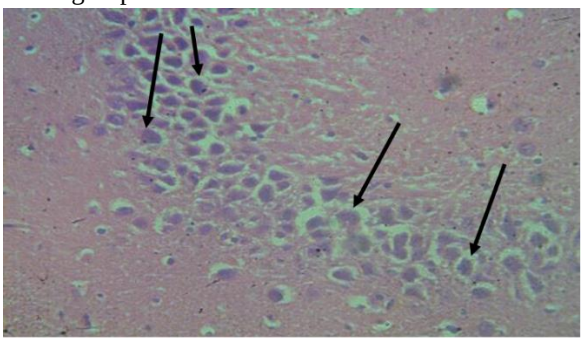

Plate 1: (H&E X400) of the hippocampus in the group I rats on day 3. Observe the normal pyramidal cells with large pyramidal nuclei (black arrows).

The result of the effects of smokeless tobacco consumption on the cytoarchitecture of the hippocampus of group II for day 3 and day 33are shown in plate 2. Slide A represents day 3 while slide B represents day 33. Observe the mild vacuolations (in day 3), chromatolysis, vacuolations and gliosis in day 33.

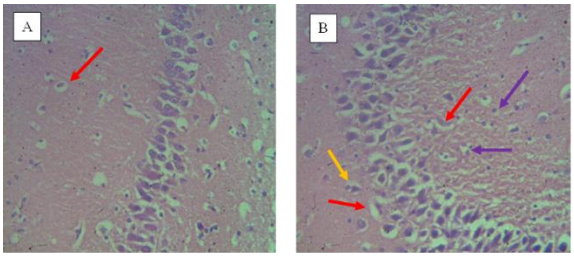

Plate 2: Micrograph (H&E X400) of the hippocampus for Group II. A is Day 3 and B is Day 33
Mild vacuolation shown by red arrows, gliosis shown by purple arrows, chromatolysis shown by orange arrow.

The result of the effects of smokeless tobacco consumption on the cytoarchitecture of hippocampus of group III for day 3 and day 33 are shown in plate 3. Slide A represents day 3 while slide B represents day 33. Observe the increased vacuolation, chromatolysis and extensive infiltration by glial cells.

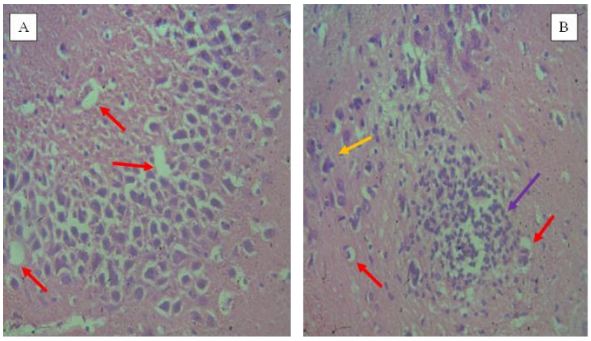

Plate 3: Micrograph (x400) of Group III showing increased vacuolation (red arrow), chromatolysis (orange arrow) and extensive infiltration by glial cells - gliosis (purple arrow) for day 3 (A) and day 33 (B)

The result of the effects of smokeless tobacco consumption on the cytoarchitecture of the hippocampus of group IV for day 3 and 33 are shown in plate 4. Slide A represents day 3 while slide B represents day 33. Observe the increased vacuolations, chromatolysis and gliosis.

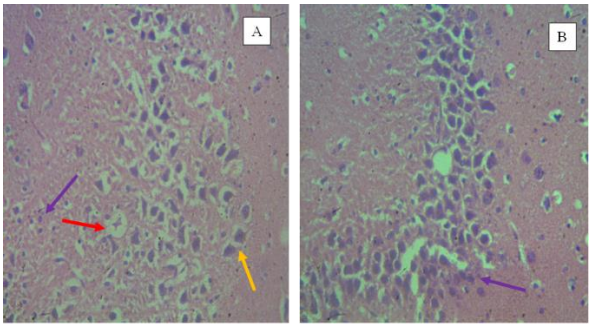

Plate 4: Micrograph (x400) of Group IV showing increased vacuolation (red arrow), chromatolysis (orange arrow) and extensive infiltration by glial cells - gliosis (purple arrow) for day 3 (A) and day 33 (B)

DISCUSSION OF FINDINGS

Several literature have been written on the effects of smokeless tobacco on the nervous system ranging from addiction, memory issues, stroke, multiple sclerosis (an autoimmune disease of the central nervous system), peripheral neuropathy, neurodegenerative disorders, etc (Patil & Singh, 2023).

In the present study, the result presented in the histomicrograph, showed a progressive histopathaological changes in the hippocampus following exposure to smokeless tobacco. Mild gliosis and mild chromatolysis were observed in the hippocampus for the group III and IVanimals at day 3. It was also accompanied by mild vacuolation. However, these features became increased at day 33 with extensive gliosis seen in the high dose group. These result present marked neurodegenerative tendencies and infiltration by glial cells (gliosis).

These observations goes in line with the work of Biswas et al. (2020), who posited that smokeless tobacco induces neuronal cell death, alteration of mitochondrial morphology, alteration of membrane potential, oxidative phosphorylation, inactivation of survival pathway and activation of apoptotic markers. These observations highlight the neurotoxic impact of prolonged usage of smokeless tobacco on the hippocampus, particularly in the CA3 region, which is crucial for cognitive functions such as learning and memory.

CONCLUSION

This study has highlighted possible progressive neurotoxic impact of prolonged usage of smokeless tobacco (snuff) on the CA3 region of the hippocampus. It has also provided reference data on the effects of smokeless tobacco consumption on the cytoarchitecture of the CA3 region of the hippocampus.

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REFERENCES

[1] S. S. Jena, S. Kabi, B. N. Panda, B. C. Kameswari, Payal, I. C. Behera, S. K. Tripathy, & S. Mahanta, - Smokeless Tobacco and Stroke - A Clinico-epidemiological Follow-up Study in A Tertiary Care Hospital. Journal of clinical and diagnostic research: JCDR, 10(10), 2016, OC40–OC43.

[2] A. Chugh, M. Arora, N. Jain, A. Vidyasagaran, A. Readshaw, A. Sheikh, J. Eckhardt, K. Siddiqi, M. Chopra, M. P. Mishu, M. Kanaan, M. A. Rahman, R. Mehrotra, R. Huque, S. Forberger, S. Dahanayake, Z. Khan, M. Boeckmann, & O. Dogar, - The global impact of tobacco control policies on smokeless tobacco use: a systematic review. The Lancet Global Health, 11(6), 2023, e953–e968.

[3] N. Sapundzhiev, and J. Werner, - Nasal snuff: historical review and health-related aspect. *The Journal of Laryngology and Otology*, 117, 2003, 686 – 691.

[4] World Health Organization: WHO, - Arsenic. https://www.who.int/news-room/fact-sheets/detail/arsenic, December 7, 2022.

[5] E. C. Aniwada, N. D. Uleanya, E. N. Ossai, E. A. Nwobi, & M. Anibueze, - Tobacco use: prevalence, pattern, and predictors, among those aged 15-49 years in Nigeria, a secondary data analysis. Tobacco Induced Diseases, 16, March 2018, 1–8.

[6] T. C. Ugwu,. & J. S. Aprioku, - Nasal snuff (Nigeria local smokeless tobacco) administration raises hepatic enzyme plasma levels in Wista rats. *European Journal of Medicinal Plants*, *16*(2), 2016, 1 – 7.

[7] P. Jha, B. Jacob, V. Gajalakshmi, P. C. Gupta, N. Dhingra, R. Kumar, - A nationally representative case-control study of smoking and death in India. N Engl J Med.358(11), 2008, 1137-1147.

[8] K. F. Rabe, S. Hurd, A. Anzueto, P. J. Barnes, S. A. Buist, P. Calverley, - Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. Am J Respir Crit Care. 176, 2007, 532-555.

[9] E. Jauniaux, G. J. Burton, - Morphological and biological effects of maternal exposure to tobacco smoke on the feto-placental unit. *Early Hum Dev*. 83(11), 2007, 699-706.

[10] O. O. Desalu, K. R. Iseh, A. B. Olokoba, F. K. Salawu, A. Danburam, - Smokeless tobacco use in adult Nigerian population. Niger J Clin Pract. 13, 2010, 382-387.

[11] J. S. Aprioku, T. C. Ugwu, - Comparative evaluation of the impact of subacute exposure of smokeless tobacco and tobacco smoke on rat testis. Int J Reprod Med, 2015, 1-10

[12] K. D. Brunnemann, D. Hoffmann, - Chemical composition of smokeless tobacco products. In: Smokeless tobacco or health. An international perspective (smoking and tobacco control monograph No. 2). Bethesda (MD), National Cancer Institute, 1992.

[13] I. Onoh, O. Owopetu, A. Olorukooba, C. Umeokonkwo, T. Dahiru, and M. Balogun, - Prevalence, patterns and correlates of smokeless tobacco use in Nigerian adults: An analysis of the Global Adult Tobacco Survey. *PLoS ONE*, 16(1), 2021, e0245114.

[14] S. N. Kabwama, D. Kadobera, and S. Ndyanabangi, - Perceptions about the harmfulness of tobacco among adults in Uganda: Findings from the 2013 Global Adult Tobacco Survey. Tob Induc Dis. 16(59), 2018, 1– 7.

[15] J. E. Henningfield, A. Radzius, and E. J. Cone, - Estimation of the available nicotine content of six smokeless tobacco products. *Tob. Control.*, 4, 1995, 57 – 61

[16] IARC Working Group on the Evaluation of Carcinogenic Risks to Humans (2012). Personal Habits and Indoor Combustions. *Lyon (FR): International Agency for Research on Cancer (IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, No. 100E.)*. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK304388/

[17] C. Loveness, D. Nabuzoka, and R. Paul, - Factors Associated with Snuff Usage and its Neurocognitive Effects among Women in Ndola

Urban, Zambia. Medical Journal of Zambia. 47(1), 2020, 25 – 32.

[18] J. Constance, J. M. Lusher, and E. Murray, - The use of smokeless tobacco among UK South Asian communities. MOJ Addiction Medicine and Therapy, 6, 2019, 49-53.

[19] P. K. Avti, S. Kumar, C. M. Pathak, K. Vaiphei, & K. L. Khanduja, - Smokeless tobacco impairs the antioxidant defence in liver, lung, and kidney of rats. *Toxicological Sciences: An official Journal of the Society of Toxicology*, *89*(2), 2006, 547 – 553.

[20] G. E. Swan and C. N. Lessov-Schlaggar, - The Effects of Tobacco Smoke and Nicotine on Cognition and the Brain. Neuropsychology Review. 17, 2007, 259-73.

[21] M. B. Yusuf, A. R. Muhammad, Z. Z. Moses, U. K. Ikechukwu, and A. M. Mustapha, - Survey of Snuff Use and Preliminary Study of Effect of Two Brands on Brain Antioxidants and Acetylcholinesterase Enzyme of Wistar Albino Rats. *Aim Journal*, 2021.

[22] R. Gupta, S. Gupta, S. Sharma, - (2020). Association of smokeless tobacco and cerebrovascular accident: a systematic review and meta-analysis of global data. *J Public Health (Oxf)*. 42, e150-e157.

[23] E. O. Earnest and D. L. Ajaghaku, - Guidelines on dosage calculation and stock solution preparation in experimental animals' studies. *Journal of Natural Sciences Research*, *4*(18), 2014, 100 – 106.

[24] T. C. Adias, A. O. Ajugwo, T. A. Erhabor, B. I. Adejumo, and C. C. Azikiwe, - Effect of sub-lethal doses of smokeless tobacco (snuff) on some haemato-rheological parameters using albino wistar rats. *American Journal of Medical Sciences and Medicine, 2*(3), 2014, 54 – 57.

[25] S. Biswas, H. Das, U. Das, A. Sengupta, R. Dey Sharma, S. C. Biswas and S. Dey, - Smokeless tobacco induces toxicity and apoptosis in neuronal cells: a mechanistic evaluation. *Free Radical Research, 54*(7), 2020, 477 – 496.

[26] J. E. Henningfield, R. V. Fant, A. Radzius, and S. Frost, - Nicotine concentration, smoke pH and whole tobacco aqueous pH of some cigar brans and types popular in the United States. *Nicotine & Tobacco Research, 1*(2), 1999, 163 – 168.

[27] R. S. Barr, D. A. Pizzagalli, M. A. Culhane, D. C. Goff and A. E. Evins, - A single dose of nicotine enhances reward responsiveness in non-smokers: implications for development of dependence. *Biological Psychiatry, 63*(11), 2008, 1061 – 1065.

[28] D. J. K. Balfour, - The neurobiology of tobacco dependence: a preclinical perspective on the role of the dopamine projections to the nucleus. *Nicotine & Tobacco Research, 6*(6), 2004, 899 – 912.

[29] A. Patil, & N. Singh, - Tobacco's toll: looking at the long-term systemic effects. *Preprints.* 2023.