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Bawa Muhammad

Department of Biochemistry and Molecular Biology, Faculty of Science, Nasarawa State University Keffi, Nigeria, rabbanimuhammad1@gmail.com

Moses Zaruwa

Nasarawa State University Keffi Faculty of Natural and applied Science Department of Biochemistry and Molecular Biology, moseszira@gmail.com

Ruqaiyatu Muhammad

Federal Medical Center Keffi Department of pharmacy, rugqatenku@gmail.com

Mustapha Ahmed

Nasarawa State University Department of Biochemistry and molecular Biology, muhammedmustapha@gmail.com

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Survey of Snuff Use and Preliminary Study of Effect of Two Brands on, Brain Antioxidants and Acetylcholinesterase Enzyme of Wister Albino Rats

Muhammad B. Yusuf^{1*} PhD, Adamu R. Muhammad² Pharm D, Zaruwa Z. Moses¹ PhD, Ubaoji K. Ikechukwu³ Ahmed M. Mustapha¹ BSc.

* *Corresponding Author:*

Muhammad, B. Yusuf
rabbanimuhammad@yahoo.com

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¹Department of Biochemistry and Molecular Biology, Faculty of Science, Nasarawa State University Keffi, Nigeria.

²Department of Pharmacy, Federal Medical Centre Keffi, Nigeria.

³Department of Applied Biochemistry, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria.

ABSTRACT

Background: The use of snuff has resurfaced globally with an increasing trend among Nigerian populace.

Aim of the work: The research aimed to survey snuff use, effect of special moringa sundu (SMS) and smokeless tobacco (ST) on brain antioxidants and acetylcholinesterase (AChE) in vivo.

Material and Methods: 300 people were interviewed using questionnaire, on the consumption rate and effects of some snuff brands. Thereafter, 30 Wister albino rats (110-120g) were randomly divided into five groups to investigate the effects of two brands in vivo. Group 1 (control) received only distilled water daily. Groups 2 and 3 (received 6mg and 3mg /kg b.w.t of ST respectively), groups 4 and 5 (received 6mg and 3mg / kg b.w.t of SMS respectively). After two months, brains of all rats were isolated, homogenized and analyzed.

Result: Survey revealed that 70 % use combination of different brands for sexual enhancement, 45 % use SMS while 15 % use ST for headache, vision, and itching. Animal studies revealed non-significant increase in SOD and GPx activities in groups 2, 3, and 4 compared to control, with concomitant increase in GSH levels. While a significant increase ($p < 0.5$) in MDA was detected in groups 2, 4 and 5. Brain AChE activity decreased significantly in both group 2 and 3 but increased significantly in group 4 and 5 compared to the control.

Conclusion: Oxidative stress is inhibited by SMS but induced by high dose ST while acetylcholinesterase activity is induced by SMS but inhibited by ST.

Keywords: Snuff; brain; antioxidants; acetylcholinesterase enzyme.

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INTRODUCTION

Snuff is any product made from ground or pulverized tobacco leaves intended to be placed in the oral or nasal cavity.¹ This distributes rapid nicotine sensation with long-lasting fragrance and essence. Snuff could be placed between lips and gum (moist snuff), or sniffed through the nose (dried powdered tobacco product). For instance "Naswar" a dipping smokeless tobacco product commonly used in Pakistan, Afghanistan, Iran and South Africa.² Snuff became common in the United Kingdom throughout the seventeenth century, but powdered tobacco is documented to have been used by native inhabitants of Brazil before the coming of the Spaniards.³ In the western world, Snuff reemerged as substitutes, for tobacco and became generally acceptable among younger generations after the ban on smoking in many public places. The traditional snuff production involves the selection of varieties of tobacco leaves,

sun-dried then subjected to fermentation process which gives a characteristic scent. It is converted to powder known as snuff blend which is mixed with calcium oxide and wood ash (original fine snuff without addition of flavors). However, most often varieties of spice, piquant, fruit, flora and menthol are added to the pure or blend snuff. The types of snuff include dry, wet, dipping tobacco, chewing tobacco, and creaming snuff.⁴

Another form of snuff made from *moringa* (drumstick) are now widely accepted and sold in some African countries including Nigeria. Although they are a blend of chiefly *moringa* leaves, they are not devoid of tobacco. *Moringa* snuffs are affectionately consumed by car drivers, motorcyclists, menial laborers and even some members of the elite. They are sold under brand names such as : Special moringa sundu, AK-47 boss, Hajiyah aisha man power, Sweet mother, Normal

tobacco, lion brand, Hajiyah Bilkisu, Hajiyah Fatima, Hajiyah Bilkisu Ma'a shaa Allah, Shehu Barhama, and Hajiyah Hauwa.

Recent studies suggested that long term use of smokeless tobacco could predispose to free radical generation and oxidative stress.^{5,6} Nicotine is a main bioactive component of tobacco. Previous studies have shown that low doses of nicotine can improve memory function and reduced plaque burden and could be used as anti-Alzheimer disease agent and improved attention performance in schizophrenia patients.^{7,8} However, some studies have indicated that nicotine impairs cognition in human and animal subjects. These inconsistencies could be attributed to the doses. It appears high dosage of nicotine might induce neurotoxicity.⁹ and excite oxidative stress, while low amount could improve cognitive performance.⁸ On the other hand, the major component of the modern West African snuff is *moringa oleifera* a plant considered as one of the most beneficial trees in the world, with several medicinal, nutritional and industrial applications.^{10,11} Moringa is rich in vitamins, antioxidants, β -carotene, amino acids, phenolics, and flavonoids.¹² These various components of moringa make potent free radical scavengers, enzyme inhibitors, antioxidants, anti-bacterial, anti-tumor, cholesterol lowering, antipyretic, anti-inflammatory, anti-diabetics⁷ anti-ulcer among others.¹³

The rate of moringa snuff consumption has rampantly increased in Nigeria particularly the Northern region; where in the past, snuffing was considered to be filthy. The addiction to snuff is obvious, although the addicts claim that, it has various therapeutic benefits against different ailments. Unlike the "traditional snuff" (i.e. a blend of purely tobacco and flavouring agent) whose effect has been studied and documented to cause disorders such as cancer (of the mouth, lips, nasal cavities, oesophagus and gut), diabetes; hypercholesterolemia, myocardial infarction and teratogenicity,⁶ modern snuff contains high percentage of *moringa* beside other adjuncts and was never studied. For this reason, the current study aimed to survey some brands of snuff and the effect of special moringa sundu (SMS) and smokeless tobacco (ST) on brain antioxidants and acetylcholinesterase (AChE) *in vivo*.

MATERIAL AND METHODS

Chemicals and kits

The chemicals used in this research include disodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4) sodium chloride (NaCl) and ethanol 95% and isoflurane were purchased from Fluka, Switzerland. All kits used were obtained from Solarbios Life Science Limited, Beijing China. These include Superoxide dismutase (SOD) Catalog Number: BC0175, Glutathione Peroxidase (GPX) Catalog Number: BC0174, Malondialdehyde (MDA) Catalog Number: BC002, reduced glutathione (GSH) Catalog Number: BC1175), Acetylcholinesterase (AChE) Assay Kit Catalog Number: BC2020

Snuff samples, composition and preparation

The products figure 1 (smokeless tobacco and special moringa sundu) were purchased from retail shop in keffi town, Nasarawa State, Nigeria. Based on manufacturer's description, Smokeless tobacco contains ground tobacco leaves and menthol while special moringa sundu contains tobacco powder, menthol and moringa powder.

To prepare snuff solution; 1g of sample was dissolved in 1 liter of deionized water and refrigerated until used.



Fig. 1: Snuff samples: A. Special Moringa Sundu; B. Smokeless Tobacco

A: Commercial product containing a mixture of moringa powder, tobacco powder and menthol

B: Commercial product containing finely pulverized dried tobacco leaves with neither sweetener nor flavourings

Experimental Design:

The study was carried out in two phases. The first phase involved the use of questionnaire to obtain relevant information from snuff users. This provided an insight into the average quantity of each snuff consumed and also the reward-seeking behavior of the users.

The second phase *i.e.* the *in vivo* study in rats which was approved by the Nasarawa State University Keffi Animal Ethics Committee, involved the use of smokeless tobacco, special moringa sundu, and thirty (30) Wister albino rats (weighing 110-120g). The snuffs were obtained from shop number nine (9) Kaduna road Angwan NTA keffi, Nasarawa state; the antioxidant and acetyl cholinesterase enzyme kit used were obtained from Solarbios Life Science Limited, Beijing China while the rats were obtained from National veterinary research institute (NVRI) VOM, Plateau state.

These rats were housed in cleaned plastic cages, bedded with clean rice husks, a 12 hour light: 12 hour dark cycle and free access to food and water ad libitum. After two (2) weeks acclimation period, the animals were weighed and randomized into five (5) designated groups of six (6) rats each: Group 1 (control received no treatment), Group 2 (received a dose of 6mg/Kg body weight smokeless tobacco), Group3 (received a dose of 3mg/Kg body weight smokeless tobacco), Group4 (received a dose of

6mg/Kg body weight special moringa sundu), and Group5 (received a dose of 3mg/Kg body weight special moringa sundu)

All animals received humane treatment; they were fed with growers mesh and allowed access to water throughout the treatment period (two months). Food gavage was used in the administration of the solution.

At the end of the experiment (administering of snuff solution) rats were weighed, sedated using 5% V/v isoflurane and oxygen 1.5L / min flow, the skulls were dissected quickly and the brain tissues were quickly removed and washed in phosphate buffer solution of pH 7.4. The brain tissues were instantly placed in ice and refrigerated at -20°C for 24hrs.

Preparation of Brain homogenate

Brain tissues were cut into pieces, weighed and two (2g) grams was homogenized in 20 volumes of phosphate buffer saline pH 7.4 (0.1M), at 700g in ice for 10min, then centrifuged for 15min at 5000 rpm. The supernatant was then collected and stored at -20 °C until use.¹⁴

Biochemical analysis

Determination of superoxide dismutase (SOD) Activity

SOD activities were determined colorimetrically according to manufacturer's (Solarbios Life Science) instructions. SOD catalyzes the conversion of superoxide molecules ($O_2^{\cdot-}$) into either molecular oxygen (O_2) or hydrogen peroxide (H_2O_2). The $O_2^{\cdot-}$ reduces nitro-blue tetrazolium to form blue formazan, which absorbs at 560 nm. SOD reacts with $O_2^{\cdot-}$ and suppresses the blue reaction. The intensity of the blue color is inversely proportional to the SOD activity.¹⁵

Glutathione Peroxidase (GPx) Activity

The Glutathione Peroxidase Activity Assay kit was used to measure glutathione peroxidase in the tissue extracts according to manufacturer's protocol. The assay is based on the oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG) which is catalyzed by GPx. In the presence of GSH, glutathione reductase and NADPH. Glutathione peroxidase activity corresponds to oxidation of NADPH to NADP. Absorbance was measured at 340 nm.¹⁶

Determination of total reduced glutathione (GSH)

Glutathione (GSH) is a natural three peptide containing sulfhydryl (SH). It is composed of glutamic acid, cysteine and glycine; it Glutathione react with 5,5'-dithiobis-2-nitrobenoic acid (DTNB) to give a yellow product that absorbs maximum ally at 412nm. The amount of GSH was determined as GSH ($\mu\text{g/g}$ tissue).¹⁷

Determination of Malondialdehyde (MDA) level

Malondialdehyde (MDA) is one of the convenient markers of lipid peroxidation. At an acidic pH and high temperature, MDA and thiobarbituric acid

(TBA) condenses to form brown red 3,5,5-trimethyl sulfamethoxazole -2,4-dione ketone with largest absorption wavelength of 532 nm. The level of MDA was calculated by the difference between the absorbance at 532 nm, 450 nm and 600 nm as instructed by the kit's manufacturer.¹⁸

Determination of acetylcholinesterase (AChE) Activity

AChE catalyzes Ach hydrolysis to generate choline, and choline reacts with 5,5'-dithiobis(2-nitrobenzoic acid) to form 5-mercapto nitrobenzoic acid (TNB) which absorbs at 412 nm. AchE activity was expressed as U/g tissue as described by the kit manufacturers.¹⁹

RESULTS

The result in table1 revealed the response of 300 active snuff users. 70 % use combination of different brands for sexual enhancement while 40 % use Special moringa sundu (SMS) for sexual enhancement. 45 % claimed SMS relieves headache, improves vision, and relieves itching, while 15 % claimed Smokeless tobacco (ST) relieves eye itching, fever and tooth ache. SMS comes in 16g container while ST comes in 14g container. From the data obtained, 75 % of users finish a container in two weeks, 15 % in three weeks and 10 % in four weeks irrespective of the brand.

LD₅₀ Determination of different snuff products.

The results of the acute toxicity studies of various snuff products showed mild toxicity, such as difficulty in breathing, shivering, tremors, anxiety and aggressive behavior. The LD₅₀ was estimated to be 2750 mg / kg body weight and 2800 mg/kg body weight for special moringa sundu and smokeless tobacco respectively.

The result revealed a non-significant ($P>0.05$) decrease in SOD and GPx activities in all treatment groups with concomitant increase in GSH levels when compared to the control. On the contrary, a significant ($P<0.05$) increase in MDA level was observed in groups 3, 4 and 5 while group 2 showed a decrease. Furthermore, the AchE activity decreased significantly ($P<0.05$) in both groups 2 and 3 but increased in group 4 and 5 significantly, when compared to the control.

PARAMETER	TOTAL NO OF RESPONDENTS n = 300 (Percentage of respondents)		
1. DEMOGRAPHICS OF RESPONDENTS			
Sex of respondents	Males		
Age distribution of users (respondents)			
35 – 45 years	190 (63.3 %)		
46 – 55 years	80 (26.7 %)		
> 55 years	30 (10 %)		
Snuff brand consumed			
Smokeless tobacco (ST)	22 (7.3 %)		
Special moringa sundu (SMS)	95 (31.7 %)		
Others (undefined sex enhancers)	183 (61 %)		
2. SNUFF USAGE			
	Smokeless tobacco (ST)	Special moringa sundu (SMS)	Others (Undefined sex enhancers)
Product package	14g	16g	14 – 16g
Frequency of use	Several times daily	Several times daily	Several times daily
Average consumption (approx. weight consumed)			
Finish a pack every week	5% (14g)	5% (16g)	Variable (according to need)
Finish a pack every two weeks	70% (7g)	70% (8g)	
Finish a pack every three weeks	15% (5g)	15% (5g)	
Finish a pack every four weeks	10%(3.5g)	10% (4g)	
Reported effects			
Relieves headache	10%	45%	Variable
Improves vision, relieves eye itching and toothache	15%	15%	Inconsistent
Enhanced sexual performance	None	40%	70%
Increase in appetite	None	10%	Inconsistent
Dizziness at initiation	None	None	90%
Diarrhoea	None	3%	Variable
Nausea and vomiting at initiation	None	None	50%

Table 1: Survey of some Snuff Brands commonly consumed in Nigeria. The results in table1 revealed about 300 people were interviewed through a questionnaire, on the consumption rate/doses, effects experiences and the two brands consumed.

GROUP	SOD (U/g tissue)	GSH (µg/g tissue)	GPx (U/g tissue)	MDA (nmol/g tissue)	AchE (U/g tissue)
GP1 (Control)	68.37±4.14 ^a	55.13±6.49 ^a	39.61±5.33 ^a	17.42±4.41 ^a	28.05±6.23 ^a
GP2 (ST-HD)	61.46±11.32 ^{ab}	91.66±6.98 ^{ab}	36.20± 3.80 ^{ab}	21.93±1.01 ^{ab}	13.81±2.30 ^b
GP3 (ST-LD)	61.92±2.59 ^{ab}	64.07±3.58 ^{ab}	34.81± 3.32 ^{ab}	27.35±7.03 ^c	15.20±4.53 ^b
GP4 (SMS-HD)	49.55±4.35 ^c	341.59±70.78 ^c	20.16±2.16 ^c	13.26±3.10 ^{ad}	48.19±11.59 ^c
GPD (SMS-LD)	28.63±3.16 ^d	503.29±108.07 ^d	16.32±2.23 ^d	3.43±1.40 ^e	39.00±5.64 ^d

Table 2: Effect of Snuff on Brain Levels of SOD, GPx, GSH, MDA and AchE Activity of Wister Albino Rats. Results are expressed as Mean ± SD, n=6 values in each column having different superscript (a, b, c, d, e) are significantly different at p<0.05. GP = Group, ST = smokeless tobacco, SMS = special moringa sundu.

DISCUSSION

The snuff users uninterruptedly use the various products for different motives especially for sexual enhancement. Other reasons include better vision, fever, headache, itching among others. Similar scenarios have been reported among men in the US, Scandinavia and particularly in Sweden, where approximately 20 % of men snuffle on daily basis.

This addiction could be attributed to the tobacco powder and menthol usually added in all brands of snuff.²⁰ While tobacco contains nicotine whose addiction had been documented, menthol which is also incorporated in the snuff was found to potentiate tobacco addiction through biological mechanisms²¹ which include; alteration of nicotinic receptors,

masking of tobacco aversive behaviours and increasing nicotine bioavailability.^{22, 23} Although users derive some therapeutic benefit, in most cases it has just become a habit, synonymous to smoking cigarette or eating kola nut. Finally if the users actually snuffle for the acclaimed therapeutic benefits, then it is expected that they should cease to continue when the ailment is cured, but they persist.

The brain is an important part of the biological system whose function helps to regulate other parts of the body. However, any injury or form of stress experienced in this organ may have severe influence on the entire organism. Several studies have shown that the mechanism of snuff action in animals is associated with production of reactive oxygen species (ROS).^{5, 6, 12, 24}

SOD detoxifies superoxide radicals to hydrogen peroxide, which can be further detoxified to water by GPx. In this study, all groups showed an increase in MDA and GSH levels with simultaneous decrease in SOD and GPx activities. This is similar to previous studies which revealed decrease in SOD, GPx and catalase in “Maras” powder (a product that contains smokeless tobacco) users.²⁴ It indicates that tobacco induces reactive oxygen species (ROS) generation and inhibit antioxidant enzymes activities at the same time. ROS can interact with cellular biomolecules principally lipids, to generate MDA and other lipids peroxide. These lipid peroxides can interact with different proteins such as enzymes to interfere with their active site thus their activities.²⁵ On the other hand in groups 4 and 5, SOD, GPx and MDA decreased drastically with concomitant increase in GSH level. Strangely, this could be attributed to the low concentration of substrate (superoxide radical) for SOD due to the free radical scavenging and antioxidant activities of *moringa oleifera* found in the SMS snuff. It has been established that *moringa* is a rich source of vitamins, amino acids and polyphenols and flavonoids.^{12, 26} It is likely that the amino acids contribute to the GSH pool and the vitamins provide coenzymes for synthesis of GSH.²⁷ Furthermore, the polyphenols and flavonoids could have scavenged the free radicals generated either *denovo* or by the metabolism of little tobacco additive in the snuff.²⁸ Conversely, the acetyl cholinesterase enzyme (AChE) activities in groups administered with smokeless tobacco (2 and 3) decreased drastically, which could improve cognitive function due to nicotine content.²⁹ Ach is one of the central neurotransmitter, which plays a significant role in memory, learning and cognition.³⁰ It relays signal from one neuron to another in the central nervous system and from neuron to muscle fiber in the peripheral nervous system. AchE is the enzyme that breaks acetylcholine to the choline and acetate.³⁰ Inhibition or decrease in AchE activities increases the concentration of Ach at the synaptic junction and allows for potentiation of the signal.³¹ This would eventually reduce the amount of choline uptake and increase the number of nicotinic receptors; which in turn will boost memory, learning and cognition in snuff users.³² Similarly the snuff contains menthol which has been reported to potentiate and promote

binding of nicotine to nicotinic receptors (nAChRs) which is an agonist of acetylcholine.³³ Conversely, rats administered special moringa sundu snuff (group 4 and 5) showed a two fold increase in AchE activity which can lead to a decrease in the concentration of Ach at the synaptic junction and consequently, a decreased cholinergic activity. This result differs from previous research which reported that pure *moringa oleifera* water extract inhibit AchE activity.³⁴ This means that although the *moringa* sundu snuff decreased lipid peroxidation and improved GSH level, it could however, decrease alertness and cognitive function.³⁵

CONCLUSION

Although Smokeless tobacco induces oxidative stress at high concentration, low dose could improve cognitive function through inhibition of acetyl cholinesterase enzyme activity. However, Special moringa sundu decreased lipid peroxidation and increased reduced glutathione level, but also increased acetyl cholinesterase enzyme activity which would cause decline in cognitive function as a result of the decreased acetylcholine pool. These could suggest that combination of moringa and tobacco would yield better snuff.

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