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Tobacco Snuff Induced Organ Weight Changes

C. I. Ugbor[1*], L. O. Okonkwo[1,2], A. O. Nwaopara[3], N. J. Duhu[4] and E. I. Odo[4]

[1]Medical Laboratory Services, Federal Teaching Hospital, Abakaliki, Ebonyi, Nigeria.
[2]Department of Medical Laboratory Science, Ambrose Alli University, Ekpoma, Nigeria.
[3]Department of Anatomy, Ambrose Alli University, Ekpoma, Nigeria.
[4]Medical Laboratory Services, Enugu State University Teaching Hospital, Parklane, Enugu, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. Author CIU was responsible for the design of this work and supply of some literature material. Author AON was responsible for organization of the manuscript with other authors. All authors CIU, LOO, AON, NJD, and EIO contributed to the completion of this study and were actively involved in the presentation of this manuscript.

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ABSTRACT

Smokeless tobacco comes in two different forms, which are 'tobacco snuff' and 'chewing tobacco'. Tobacco snuff is the powdered form blended with potash as the main additive in Nigeria. This eight-week study was designed to investigate the effect of tobacco snuff consumption on organ weight. A total of (42) Adult Wistar rats weighing 150-300g were involved. They were divided into four groups; group A serving as control, while groups B, C and D served as the test groups. The test groups were further divided into four groups (B1, C1, D1; B2, C2, D2; B3, C3, D3; and B4, C4, D4) representing four experimental phases/duration of 2, 4, 6 and 8 weeks respectively. The rats were fed with varying doses of tobacco snuff and at the end of every 2 weeks; three randomly selected rats were prepared for organ harvest followed by organ weight measurement. The results showed statistically significant organ weight changes throughout the study. Heart, liver, lungs, spleen, small intestine, right and left kidney and right and left testis all presented organ weight loss when test groups were compared with the control. Brian showed both increase and decrease

**Corresponding author: E-mail: i.chimaresearch2@gmail.com;*

weight changes that were duration dependent when test groups were compared with the control (1.83 ± 0.18) throughout the study. Based on the existing facts, our findings support the assertion that smokeless tobacco is not safe and has the capabilities of inducing intracellular damages due to its innate and acquired deleterious effects.

Keywords: Tobacco snuff; nicotine; organ weight; rat.

1. INTRODUCTION

Tobacco consumption is a problem worldwide and the recent increase in the consumption of smokeless tobacco products (snuff and chewing tobacco) has stimulated interest into the carcinogenic effects of these forms of tobacco [1]. Scientific facts prove that tobacco snuff contains nicotine which is toxic in addition to other elements such as Natron [2,3,4,5,6,7,8]. Interestingly, the harmful effect of smokeless tobacco appears overwhelming due to various implicating scientific findings. As regards to this, [9] report that oral cells, peritoneal macrophages, and hepatic mitochondria and microsomes produce reactive oxygen species following *in vitro* incubation with an aqueous extract of smokeless tobacco which causes most of the cellular degeneration in vivo. Also, smokeless tobacco has proven to be destructive to the genetic materials in the liver, kidney and lungs [10].

Specifically, [11] contradicted the general assertion that smokeless tobacco is a safe substitute to smoking due to the result of a quantitative study on the level of carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in smokeless tobacco users. Like other components of smokeless tobacco, NNK has been implicated with lung tumors [12] among other harmful effect. Recently, [13,14] implicated tobacco snuff with organ and systemic damage. Emphatically, nicotine sustains tobacco addiction, which in turn causes devastating health problems including heart disease, lung disease, and cancer [15]. These negative health effects are believed to be caused by a number of toxicants and carcinogens present in smokeless tobacco [16,17,18].

Available literatures demonstrates that smokeless tobacco are known potential generators of free radicals which highly reactive radicals and reactive oxygen species (ROS) act as initiators of carcinogenesis, cause DNA damage, activate pro-carcinogens and alter the cellular antioxidant defence system [19,14]. Changing the balance towards an increase in the pro-oxidants over the capacity of the antioxidants is defined as oxidative stress, which might lead to oxidative damage. Due to the fact that organs are the engine house of the body and are vulnerable to toxic substances, there is a need to draw the attention of consumers to the hazardous effects and subsequent health implications of excessive tobacco snuff consumption [13]; hence this study investigates the effect of tobacco snuff consumption on organ weights.

2. MATERIALS AND METHODS

2.1 Experimental Animals

Forty two adult Wistar rats of comparable sizes and weighing (150-300g) were purchased from the animal farm of Anthonio Research Center, Ekpoma, Edo state, Nigeria. They were transferred to the experimental site where they were allowed two weeks of acclimatization in a wooden wire mesh cages under standard laboratory procedure. Overall, the animals were handled in accordance with the standard guide for the care and use of laboratory animals [20].

2.2 Substance of Study

Dry leaves of tobacco and potash were purchased from Ogbete main market, Enugu state, Nigeria. The tobacco leaves were authenticated by a botanist in the Department of Botany, Ambrose Alli University, Ekpoma, Edo state, Nigeria.

2.3 Substance Preparation

The tobacco leaves and potash were blended into powder using a mortar and iron pestle and then stored prior to the study. The blended tobacco leaves with potash were weighed using an electronic balance (Denver Company, USA, 200398. IREV. CXP-3000) to obtain the various required doses. For the purpose of this study, feed pellets were prepared as described by Nwaopara et al. [21].

2.4 Animal Grouping

The experiment involved four stages: stage 1, which lasted for a period of 2 weeks; stage 2, which lasted for a period of 4 weeks; stage 3, which lasted for a period of 6 weeks; and stage 4, which lasted for a period of 8 weeks. The rats were divided into four groups (A, B, C and D) with group A serving as control, while groups B, C and D served as the test groups. The test groups were further divided into four groups (B1, C1, D1; B2, C2, D2; B3, C3, D3; and B4, C4, D4) representing four experimental phases/duration (2, 4, 6 and 8 weeks) and varying doses of tobacco dust mixed with potash respectively. At the end of 2, 4, 6 and 8 weeks respectively, 3 randomly selected rats from the groups were prepared for organ harvest.

2.5 Study Duration

The preliminary studies, animal acclimatization, substance procurement (tobacco leaves and potash), actual animal experiment and evaluation of results, lasted for five months. However, the actual administration of oral tobacco dust and potash (tobacco snuff) to the test animals lasted for 8 weeks (2weeks, 4weeks, 6weeks and 8 weeks respectively).

2.6 Substance Administration

In phase 1 (2 weeks), group A (control) received 100 g of feed and distilled water only whereas test group B, C and D received 97.12 g of feed, 2.4 g of tobacco dust and 0.48 g of potash**;** 94.24g of feed, 4.80 g of tobacco dust and 0.96 g of potash**;** and 91.36 g of feed, 7.20 g of tobacco dust and 1.44 g of potash respectively. Each test group received distilled water *ad libitum*.

In phase 2 (4 weeks), group A (control) received 75 g of feed and distilled water only, whereas test group B, C and D received 72.84 g of feed, 1.8 g of tobacco dust and 0.36 g potash**;** 70.68 g of feed, 3.6 g of tobacco dust and 0.72g of potash**;** and 68.52 g of feed, 5.4 g of tobacco dust and 1.08 g of potash respectively. Each test group received distilled water *ad libitum*.

In phase 3 (6 weeks), group A (control) received 50g of feed and distilled water only, whereas test group B3, C3 and D3 received 48.56 g of feed, 1.2 g of tobacco dust and 0.24 g potash; 47.12 g of feed, 2.4 g of tobacco dust and 0.48 g of potash; and 45.68 g of feed, 3.6 g of tobacco dust and 0.72 g of potash respectively. Each test group received distilled water *ad libitum*.

In phase 4 (8 weeks), group A (control) received 25 g of feed and distilled water only, whereas test group B4, C4 and D4 received 24.28 g of feed, 0.6 g of tobacco dust and 0.12 g potash; 23.56 g of feed, 1.2 g of tobacco dust and 0.24 g of potash; and 22.84 g of feed, 1.8 g of tobacco dust and 0.36 g of potash respectively. Each test group received distilled water *ad libitum*.

The concentrations of tobacco used in this study were deduced from the work of Bagchi et al. [72] while that of potash was deduced from Ugbor et al. [22].

2.7 Sample Collection

At the end of each stage of the experiment, the animals were scarified to obtain selected organs for measurement using the electric balance (Denver Company USA 200398). The average weights were determined and recorded.

2.8 Data Analysis

All the data collected were subjected to statistical analysis using SPSS (version 18). The test groups' values were compared with the control using ANOVA (LSD) at 95% level of confidence.

3. RESULTS

The organ-weight values are as presented in Tables 1-11. Table 1 represents the effect of tobacco snuff consumption on heart organ weight (g). The heart organ weight of the test groups showed statistical significant difference ($P \leq 0.05$) from the values of the control (0.87 ± 0.17 g) throughout the experimental period of 2 weeks, 4 weeks, 6 weeks and 8 weeks except in group D 2weeks. However, gradual but severe steady myocardial organ shrinkage in a dosage and duration dependent manner was observed.

Table 2 represents the effect of tobacco snuff consumption on brain weight (g) of the experimental animals and control. The brain weight of the tests showed statistical significant difference ($P \leq 0.05$) from the values of the control (1.83 ± 0.18 g) in group C and D at 2 weeks period of the experiment. Also, the entire test groups presented irregular changes in weight value as compared to the control, though not statistical significant.

Table 3 represents the effect of tobacco snuff consumption on liver weight (g) of the experimental animals and control. The liver weight of the tests showed statistical significant difference (P≤0.05) from the values of the control (8.29 ± 1.38 g) throughout the experimental period. The liver organ weight presented severe weight reduction that is dosage dependent.

For Tables 4, 5 and 6, the effect of tobacco snuff consumption on lungs, spleen and small intestine showed statistically significant difference when test groups were compared with the control. In the result, the weight values for lungs presented significant differences that are dosage dependent except for group D 2 weeks that showed no significant difference. Spleen weights showed significant difference throughout the study except for group C (6 and 8 weeks), and group D (4 and 8 weeks). In the case of small intestine, group B presented no significant difference throughout the study. Group C showed significant difference at (6 and 8 weeks) and non significant difference at (2 and 4 weeks). For group D, there was significant decrease at (2 and 4 weeks) and non significant decrease at (6 and 8 weeks).

Table 1. The effects of tobacco snuff consumption on organ weight of rats

Organ weighed (g)		Control group A	Test groups		
			B	C	D
Heart	2 weeks	0.87 ± 0.17^{a}	0.60 ± 0.03^{b}	0.61 ± 0.11^{b}	0.71 ± 0.19^{a}
	4 weeks	0.87 ± 0.17^{a}	0.64 ± 0.06^{b}	0.51 ± 0.11^{b}	0.51 ± 0.08^{b}
	6 weeks	0.87 ± 0.17^{a}	0.66 ± 0.12^{b}	0.59 ± 0.12^{b}	0.51 ± 0.11^{b}
	8 weeks	0.87 ± 0.17^{a}	0.57 ± 0.06^{b}	0.55 ± 0.06^{b}	0.47 ± 0.02^{b}

All the values of the test groups with different subscript from the controls are significantly different at p≤0.05

Table 2. The effects of tobacco snuff consumption on organ weight of rats

Organ weighed (g)		Control group A	Test groups		
			B	C	D
Brain	2 weeks	1.83 ± 0.18^{a}	1.90 ± 0.07^{a}	2.18 ± 0.06^{b}	2.19 ± 0.11^{b}
	4 weeks	1.83 ± 0.18^{a}	1.92 ± 0.15^{a}	1.75 ± 0.06^{a}	1.77 ± 0.10^{a}
	6 weeks	1.83 ± 0.18^{a}	1.82 ± 0.19^{a}	1.65 ± 0.04^{a}	1.83 ± 0.80^{a}
	8 weeks	1.83 ± 0.18^{a}	1.70 ± 0.07^{a}	1.72 ± 0.11^{a}	1.81 ± 0.14^{a}

All the values of the test groups with different subscript from the controls are significantly different at p≤0.05

Table 3. The effects of tobacco snuff consumption on organ weight of rats

Organ weighed (g)		Control group A	Test groups		
			B	C	D
Liver	2 weeks	8.29 ± 1.38^{a}	4.43 ± 0.46^{ab}	6.15 ± 0.45^{b}	5.67 ± 0.74^{b}
	4 weeks	8.29 ± 1.38^{a}	4.83 ± 0.43^{b}	4.22 ± 0.41^{b}	3.89 ± 0.38^{b}
	6 weeks	8.29 ± 1.38^{a}	5.62 ± 0.90^{b}	4.78 ± 1.22^{ab}	5.11 ± 0.30^{b}
	8 weeks	8.29 ± 1.38^{a}	5.50 ± 0.67^{b}	6.08 ± 0.72^{ab}	4.70 ± 0.65^{b}

All the values of the test groups with different subscript from the controls are significantly different at p≤0.05

Table 4. The effects of tobacco snuff consumption on organ weight of rats

Organ weighed (g)		Control group A	Test groups		
			B	C	D
Lungs	2 weeks	1.41 ± 0.12^{a}	1.17 ± 0.02^{b}	1.62 ± 0.17^{ab}	1.60 ± 0.17^{a}
	4 weeks	1.41 ± 0.12^{a}	1.19 ± 0.02^{ab}	0.89 ± 0.12^{b}	0.94 ± 0.05^{b}
	6 weeks	1.41 ± 0.12^{a}	1.18 ± 0.35^{b}	1.00 ± 0.16^{b}	1.02 ± 0.80^{b}
	8 weeks	1.41 ± 0.12^{a}	1.11 ± 0.20^{ab}	1.12 ± 0.20^{b}	1.14 ± 0.15^{b}

All the values of the test groups with different subscript from the controls are significantly different at p≤0.05

Tables 7 and 8 showed the effect of tobacco snuff consumption on kidney weight (g). The right kidney of the test groups showed statistical significant difference (P≤0.05) from the values of the control (0.75 ± 0.22 g) throughout the experimental period of 2 weeks, 4 weeks, 6 weeks and 8 weeks except in group C and D 2 weeks. Also, the left kidney presented statistical significant difference (P≤0.05) from the values of the control (0.81 ± 0.24 g) throughout the experimental period, except in group C and D 2 weeks.

For Tables 9, 10 and 11, the effect of tobacco snuff consumption on the testes showed statistically significant difference when test groups were compared with the control. In the result, the weight values for the testes presented significant differences that are dosage dependent throughout the study.

Table 5. The effects of tobacco snuff consumption on organ weight of rats

Organ weighed (g)		Control group A	Test groups		
			B	C	D
Spleen	2weeks	0.79 ± 0.20^{a}	0.42 ± 0.30^{b}	0.43 ± 0.08^{b}	0.55 ± 0.18^{b}
	4weeks	0.79 ± 0.20^{a}	0.53 ± 0.21^{b}	0.48 ± 0.25^{b}	0.58 ± 0.03^{a}
	6weeks	0.79 ± 0.20^{a}	0.50 ± 0.08^{b}	0.57 ± 0.25^{a}	0.50 ±0.17^{b}
	8weeks	0.79 ± 0.20^{a}	0.53 ± 0.17^{b}	0.69 ± 0.19^{a}	0.56 ± 0.10^{a}

All the values of the test groups with different subscript from the controls are significantly different at p≤0.05

Table 6. The effects of tobacco snuff consumption on organ weight of rats

Organ weighed (g)		Control group A	Test groups		
			B	C	D
Small intestine	2weeks	17.03 ± 3.08^{a}	14.74 ± 2.43^{a}	16.79 ± 4.06^{a}	10.40 ± 0.91^{b}
	4weeks	17.03 ± 3.08^{a}	13.09 ± 0.79^{a}	14.82 ± 6.00^{a}	11.15 ± 1.42^{b}
	6weeks	17.03 ± 3.08^{a}	14.63 ± 3.43^{a}	11.59 ± 3.47^{b}	14.28 ± 1.51^{a}
	8weeks	17.03 ± 3.08^{a}	13.85 ± 0.46^{a}	18.79 ± 3.25^{ab}	13.63 ± 0.19^{a}

All the values of the test groups with different subscript from the controls are significantly different at p≤0.05

Table 7. The effects of tobacco snuff consumption on organ weight of rats

Organ weighed (g)		Control group A	Test groups		
			B	C	D
Rt. Kidney	2weeks	0.75 ± 0.22^{a}	0.44 ± 0.01^{b}	0.70 ± 0.07^{a}	0.63 ± 0.01^{a}
	4weeks	0.75 ± 0.22^{a}	0.52 ± 0.12^{b}	0.46 ± 0.03^{b}	0.44 ± 0.02^{b}
	6weeks	0.75 ± 0.22^{a}	0.53 ± 0.13^{b}	0.46 ± 0.11^{b}	0.48 ± 0.08^{b}
	8weeks	0.75 ± 0.22^{a}	0.56 ± 0.17^{b}	0.45 ± 0.15^{b}	0.51 ± 0.03^{b}

All the values of the test groups with different subscript from the controls are significantly different at p≤0.05

Table 8. The effects of tobacco snuff consumption on organ weight of rats

Organ weighed (g)		Control group A	Test groups		
			B	C	D
Lt. Kidney	2weeks	0.81 ± 0.24^{a}	0.50 ± 0.12^{b}	0.59 ± 0.10^{a}	0.69 ± 0.05^{a}
	4weeks	0.81 ± 0.24^{a}	0.56 ± 0.06^{b}	0.43 ± 0.04^{b}	0.45 ± 0.01^{b}
	6weeks	0.81 ± 0.24^{a}	0.60 ± 0.09^{b}	0.63 ± 0.22^{b}	0.48 ± 0.04^{b}
	8weeks	0.84 ± 0.24^{a}	0.66 ± 0.13^{b}	0.66 ± 0.10^{b}	0.51 ± 0.10^{b}

All the values of the test groups with different subscript from the controls are significantly different at p≤0.05

Table 9. The effects of tobacco snuff consumption on organ weight of rats

Organ weighed (g)		Control group A		B		C	
		T_1	T_2	T_1	T_2	T_1	T_2
Testes (T)	4weeks	1.84±0.24^{a}	1.82± 0.42^{a}	1.53±0.92^{a}	1.61± 0.76^{a}	0.77±0.14^{b}	0.82± 0.12^{b}

Table 10. The effects of tobacco snuff consumption on organ weight of rats

Organ weighed (g)		Control group A		C		D	
		T_1	T_2	T_1	T_2	T_1	T_2
Testes	6weeks	1.84 ±0.24[a]	1.82 ± 0.42[a]	0.81 ± 0.51[b]	0.96 ± 0.36[b]	0.56 ± 0.27[b]	0.51 ± 0.22[b]

Table 11. The effects of tobacco snuff consumption on organ weight of rats

Organ weighed (g)		Control group A		B		D	
		T_1	T_2	T_1	T_2	T_1	T_2
Testes	8weeks	1.84±0.24[a]	1.82 ± 0.42[a]	1.32 ± 0.24[a]	1.40 ±0.24[a]	0.51±0.20[b]	0.52 ± 0.23[b]

4. DISCUSSION

Specifically, much attention has been given to the quantitative aspects of food ingestion in relation to performance and metabolic function [23,24,25]; relatively few concerns have been geared towards the systemic effects of smokeless tobacco consumption. Recently, the increase in the consumption of smokeless tobacco products (snuff and chewing tobacco) has stimulated interest into the carcinogenic effects of these forms of tobacco [1]. As a recreational drug, tobacco snuff has been implicated with several systemic and organ damage [13,14]. Substance such as yaji [26], Alomo bitters [27] and Xylopia aethiopica [28] have been known to cause pathologic organ and body weight changes. Interestingly, the result of this study presented organ weight alterations that are dosage and duration dependent and this is linked to the potentials of the active ingredients in tobacco snuff. Ever more, heart organ showed mild to severe weight loss which could be due to the established devastating cardiovascular effect of smokeless tobacco Several authors [29,30,31,32,33] reported that the cardiovascular risk of smokeless tobacco are the same with that of cigarette smoking. Also, the injurious effects of smokeless tobacco to the heart tissue could be due to its additive such as 'natron' which [34] has reported to have contributed to the incidence of peripartal cardiac failure in Zaria and Malumfashi areas of Northern Nigeria. In the same vein, [35,36] disclosed that tobacco use is associated with the development of severe atherosclerosis possibly via mechanisms involving increased oxidative stress and nitric oxide (NO) inactivation in the vascular endothelium. Inclusively, [37] discovered that smokeless tobacco also induces cardiovascular impairment via endothelial dysfunction involving flow-mediated dilatation (FMD), high-sensitive C- reactive protein (hsCRP) and homocysteine alterations. [38] implicated smokeless tobacco in the development of cardiovascular diseases, peripheral vascular disease, hypertension, peptic ulcers, and fetal morbidity and mortality.

Molecular biology studies suggest that the α_4 β_2 nicotinic acetylcholine receptor subtype is the main receptor mediating nicotine dependence. Nicotine acts on these brain nicotinic cholinergic receptors to facilitate neurotransmitter release (dopamine and others), producing pleasure, stimulation, and mood modulation [15]. In the other hand, the result on brain weight potentiates possible deleterious effects which could be due to exicitotoxicity. In a previous study, [39] revealed smokeless tobacco induced cirrhosis of the liver. Also, [1,10] showed that histological findings of smokeless tobacco extract revealed inflammation and degeneration of the liver hepatocytes and blockage of liver sinusoids. Ugbor et al. [14] reported tobacco snuff induced severe hepatic alterations and possible tobacco hepatitis and these findings are in line with the result this study.

The lungs are the organ that regulates gaseous exchange in and out of the human system and can be plagued by some dysfunctions [40]. Due to the sensitivity of the lungs to exogenous air particles, [41] reported tobacco snuff induced progressive lung function impairment. It is discovered that inhalation of potentially toxic materials in the work places can lead to major lung diseases [2,3,4,5,7,8] and tobacco snuff is known to contain nicotine which is toxic in addition to other elements. Implicatively, [42] reported increased mortality from oral and pharyngeal cancers in a case-controlled study of use of snuff. As known, the types of smokeless tobacco products used around the world vary according to region, as do the health risks associated with them [43] and the health implications of tobacco use range from various chronic diseases to death attributable to direct or passive smoking and smokeless tobacco use [44]. The effect of tobacco use to the spleen is

not clear, though the result of this study shows possible organ degeneration.

Generally, smokeless tobacco has been associated with periodontal disease [45,46], precancerous oral lesions [47], oral cancer [48], and cancer of the kidney [49,50], as well as pancreas [51], and digestive system pathogenesis [52]. According to Mitchell et al. [44], smokeless tobacco has been implicated in gastro-oesophageal reflux disease, peptic ulcer and inflammatory bowel disease. More so, in a case-control study in Mizoram, India, it was discovered that the risk for gastric cancer was more for tobacco chewers [53]. Literatures have it that smokeless tobacco like tobacco smoke contains a variety of carcinogen including *N*-nitroso compounds and nitrogen oxides that may promote endogenous formation of *N*-nitroso compounds [54], which have been linked to gastric carcinogenesis [55] and this could be the cause of stomach organ atrophy observed in this study. Moreover, there is sufficient evidence that smokeless tobacco causes oral and pancreatic cancer in humans and sufficient evidence of carcinogenicity from animal studies [56].

The observed changes in kidney weight (left and right) are in line with the reports by Pramod et al. [19] and Gonzalez [57], who stated that aqueous extract of smokeless tobacco, impairs enzymatic antioxidant defense system and induces oxidative stress/lipid peroxidation in liver, lung, and kidney. Already, this oxidative stress–induced lipid peroxidation, according to Gonzalez [57] and Pramod et al. [19], has been implicated in malignant transformation. More so, this oxidative stress which has been established as known cytotoxic agent could be the cause of kidney organ shrinkage due to its degenerative effect. It is known however, that elevated creatinine level is associated with abnormal renal function, especially glomerular function [58,59,22] reported smokeless tobacco induced (tobacco snuff) renal toxicity. Due to the fragile nature of the nephrons to toxicity, the result of this study indicates renotoxicity with intracellular degeneration. Although, without doubt [60,61] had earlier stated that a progressive kidney failure can be associated with a gradual decrease of renal and non-renal elimination of nicotine, and this potentiates nephrotoxicity. Also, the effects of heavy metals in tobacco and heavy metals like Cadmium (Cd), Mercury (Hg) and Lead (Pb), might be another possible mechanism for tobacco-induced renal damage [62,63,64,65]. The male reproductive system is known to be highly sensitive to many chemicals and drugs which have been found to pose adverse effects on male reproductive capacity under certain conditions [66] and with smokeless tobacco having many harmful components, the resultant decrease in testicular weight showed potential toxic effect. The decrease in testicular weight observed in this study opposed the fact that increase in serum testosterone or treatment with androgens is associated with increased secretory activity and increased organ weight [67,68,69,70,71]. Conclusively, due to the fact that many diseases are secondary to different unknown causative factors, and our society is filled with so many uncommon diseases, which are more likely to be caused by uncontrolled consumption of some substances, there is need for more pro-active measures as tobacco snuff have been found to possess both innate and acquired deleterious traits.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee".

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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