

# The effect of sub-lethal doses of smokeless tobacco (snuff) on certain hematological and hemostatic parameters in Wistar rats

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## Abstract

**Objective:** We investigated the effect of sub-lethal concentrations of orogastrically-administered tobacco on some hematological and hemostatic parameters of Wistar rats.

**Methods:** Twenty young male Wistar rats with weights between 170-220 g were used for the study. The oral LD50 for the tobacco snuff concentration was determined as 10 mg per 200 g rat body weight. The control group A was given 1 ml of distilled water, while the experimental groups (B, C, D) were administered with tobacco snuff concentrations of 4 mg, 6 mg, and 8mg per 200 g body weight, respectively, through an oral route with the aid of orogastric tube for 6 weeks. Blood samples were collected to estimate hematocrit; red blood cell (RBC), white blood cell (WBC), and platelet counts; bleeding time and blood clotting time.

**Results:** When compared with the control group, our data indicated significantly higher total WBC in groups C and D ( $P < 0.05$  and  $P < 0.001$ , respectively); lower RBC in group D ( $P < 0.05$ ); lower platelet counts in all the experimental groups ( $P < 0.001$ ); and higher blood clotting time in groups C and D ( $P < 0.05$  and  $P < 0.001$ , respectively). However, no significant effects of snuff consumption were observed on the experimental groups with regard to hematocrit and bleeding time.

**Conclusion:** Our findings indicated that chronic tobacco consumption at higher sub-lethal levels may put the body at some risk of adverse hematological and hemostatic conditions.

## Key words:

Hematological parameters; Hemostatic variables; Smokeless tobacco; Tobacco snuff; Wistar rats

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## Introduction

Tobacco is the dried and processed leaves of the plant *Nicotiana tabacum* that is widely cultivated and commercially grown in many countries of the world. It is mostly consumed in the forms of smoking, chewing, snuffing, or dipping tobacco. Its usage is an activity that is practiced by some 1.1 billion people, and up to one-third of the adult population [1]. The effect of smokeless tobacco use on health irrespective of the mode of consumption has been well documented. The risks associated with smokeless tobacco use include cancer (particularly oral, esophageal and pancreatic cancers), heart disease, gum disease and oral lesions other than cancer [2]. The World Health Organization estimates that the use of tobacco caused 5.4 million deaths in 2004 [3] and 100 million deaths over the course of the 20<sup>th</sup> century [4]. Similarly, the United States Center for Disease Control and Prevention describes tobacco use as "the single most important preventable risk to

human health in developed countries and an important cause of premature death worldwide"[5].

People in many regions and countries, including North America, northern Europe, India and other Asian countries, and parts of Africa, have a long history of using smokeless tobacco products. In Nigeria, particularly, tobacco snuff is utilized for cultural and traditional purposes. It is either inhaled through the nose or applied orally. Some addicts also chew the dried leaves. The frequency and speed of tobacco consumption and the effects which it has on the body is directly related to nicotine, the most prominent phytochemical found in tobacco. All tobacco products, including smokeless tobacco, contain nicotine, which is addictive [2]. A Center for Disease Control and Prevention study of the 40 most widely used popular brands of moist snuff showed that the amount of nicotine per gram of tobacco ranged from 4.4 to 25 milligrams [6]. Other studies have

shown that moist snuff had between 4.7 and 24.3 milligrams, dry snuff had between 10.5 and 24.8 milligrams, and chewing tobacco had between 3.4 and 39.7 milligrams per gram of tobacco [7].

Users of smokeless tobacco and users of cigarettes have comparable levels of nicotine in the blood. In users of smokeless tobacco, nicotine is absorbed through the mouth tissues directly into the blood, where it goes to the brain. Even after the tobacco is removed from the mouth, nicotine continues to be absorbed into the bloodstream. In addition, the nicotine stays in the blood longer for users of smokeless tobacco than for smokers [8]. The level of nicotine in the blood depends on the amount of nicotine in the smokeless tobacco product, the tobacco cut size, the product's pH (a measure of its acidity or basicity), and other factors [6].

The status of hematological and hemostatic parameters, are useful physiological markers of organ and tissue damage and dysfunction. In view of the various pharmacological actions of nicotine and additives and the wide use in many regions and countries, chronic consumption of tobacco snuff may affect the status of hematological and hemostatic parameters and further delineate the effects of tobacco use to health. However, there are limited studies on the effect of consumption of tobacco snuff on hematological and hemostatic variables in both man and animals. In the present study we investigated the effect of sub-lethal doses of tobacco snuff on hematological parameters such as hematocrit; red blood cell (RBC), white blood cell (WBC) and platelet counts; and hemostatic parameters such as bleeding time and blood clotting time in Wistar rats after a six-week exposure period.

## Materials and Methods

### *Experimental Animals*

Twenty young male Wistar rats of weights between 170 to 220 g were procured from Department of Agriculture University of Nigeria, Nsukka, Enugu state, Nigeria. The animals were taken to the animal house of the Department of Anatomy, Anambra State University, Uli, Nigeria where they were kept in a big cage of four compartments with stainless guaze on top for ventilation. They were maintained on standard feed obtained from Agro Feed Mills Nig. Ltd. and were allowed access to water *ad libitum* throughout the period of the experiment. The experimental animal house was maintained at a temperature range of

32-37°C. All animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health [9]. The Research and Ethical Committee of the Faculty of Basic Medical Sciences of Anambra State University approved the study.

### *Tobacco snuff dose preparation*

We used commercially prepared tobacco snuff in this study bought from Maxwell Nig. Ltd., Enugu State. The snuff was put in a plastic container and stored in a cool place. The lethal dose (LD50) concentration of the tobacco snuff was calculated as 10 mg/200 g rat body weight (b.w) using LD50 for nicotine in rats (50 mg/kg b.w) as standard [10]. The concentrations of tobacco snuff used were calculated as 40% LD50, 60% LD50, and 80% LD50.

### *Animal treatment*

The present experiment was designed to be dose-dependent with the experimental groups exposed to different sub-lethal doses of tobacco snuff concentrations for 6 weeks. The animals were randomly divided into four groups (A, B, C and D) of five rats each. The animals in the control group A were administered 1 ml of distilled water for the six weeks of the experiment. 4 mg, 6 mg, and 8 mg per 200 g b.w were measured and homogenously mixed in 1 ml of distilled water and administered orogastrically to the experimental groups B, C and D, respectively [11]. At the end of the 6 weeks, the animals were sacrificed by chloroform inhalation method.

### *Blood sample collection*

In order to avoid diurnal variation, blood was collected in the morning time. The blood samples used for the determination of hematocrit, RBC count, WBC count and platelet count were obtained by cardiac puncture using a sterile surgical syringe and stored into heparinized specimen containers. The blood sample used for the determination of bleeding time was obtained by piercing the ear lobe with a sterile lancet, whereas that of clotting time was obtained by cutting the tail of the rat at a distance of 1 cm from the tip of the tail. The estimation of hematocrit was done using the method as described by Alexandra and Griffiths, [12] using the microhematocrit centrifuge (Hawksley, England). The red blood cell count (Hayem's fluid) and white blood cell count (Turk's fluid) were estimated by the methods described by Dacie and Lewis [13] using the improved Neubauer counting chamber (Gelman, England). The platelet count (Rees and Ecker's solution) were estimated

by the methods described by Pal and Pal [14]. The bleeding time and the blood clotting time were measured using the Ivy's and Lee and White's methods, respectively, as described by Dacie and Lewis [13].

### Statistical analysis

Descriptive data are expressed as means and standard error of the mean (SEM). Comparative analysis between the control group and the experimental groups was performed using ANOVA (Bonferoni post-hoc test). All statistics were done using SPSS for Windows (Version 16.0). Statistical significance was set at  $P < 0.05$ .

### Results

The entire results of the study are presented in Figs.1-6.

Data indicated insignificant decrease ( $P > 0.05$ ) in hematocrit values for the experimental groups B ( $40.8 \pm 0.75\%$ ), C ( $40.5 \pm 1.44\%$ ) and D ( $42.5 \pm 2.40\%$ ) when compared to the control group A ( $42.8 \pm 1.89\%$ ) (Fig.1).

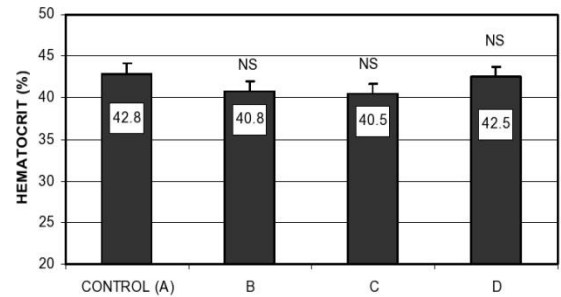
Lower RBC counts were seen in group D ( $2.2 \pm 0.43 \times 10^6$  cells/mm<sup>3</sup>;  $P < 0.05$ ), when compared to controls ( $2.7 \pm 0.09 \times 10^6$  cells/mm<sup>3</sup>). No significance ( $P > 0.05$ ) was observed in RBC count between the control group and groups B ( $2.8 \pm 0.25 \times 10^6$  cells/mm<sup>3</sup>) and C ( $2.5 \pm 0.25 \times 10^6$  cells/mm<sup>3</sup>), respectively (Fig.2).

Our result also indicated significantly higher total WBC count in groups C ( $5.9 \pm 2.07 \times 10^3$  cells/mm<sup>3</sup>;  $P < 0.05$ ) and D ( $6.5 \pm 1.50 \times 10^3$  cells/mm<sup>3</sup>;  $P < 0.001$ ) when compared to control group A ( $3.4 \pm 0.34 \times 10^3$  cells/mm<sup>3</sup>). On the other hand, no significant difference was observed between group B ( $3.8 \pm 0.54 \times 10^3$  cells/mm<sup>3</sup>) and the control group in WBC count ( $P = 0.72$ ) (Fig.3).

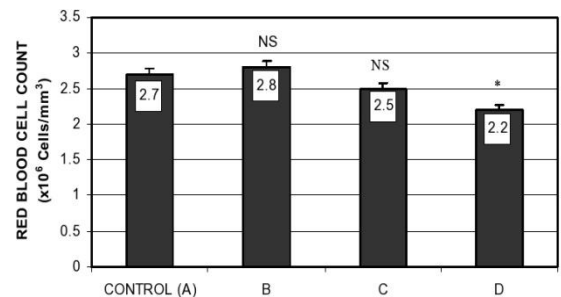
The platelet count indicated significantly lower values in all the three experimental groups ( $P < 0.001$  for all): B ( $1.3 \pm 0.18 \times 10^5$  cells/mm<sup>3</sup>), C ( $1.2 \pm 0.10 \times 10^5$  cells/mm<sup>3</sup>), D ( $1.0 \pm 0.12 \times 10^5$  cells/mm<sup>3</sup>) vs. control group A ( $1.9 \pm 0.18 \times 10^5$  cells/mm<sup>3</sup>) (Fig.4).

Clotting time was significantly higher in groups C ( $2.0 \pm 0.17$  min;  $P < 0.05$ ) and D ( $2.4 \pm 0.10$  min;  $P < 0.001$ ) than the control group ( $1.4 \pm 0.14$  min) but not significantly differing in group B ( $1.5 \pm 0.08$  min;  $P > 0.05$ ) (Fig.5).

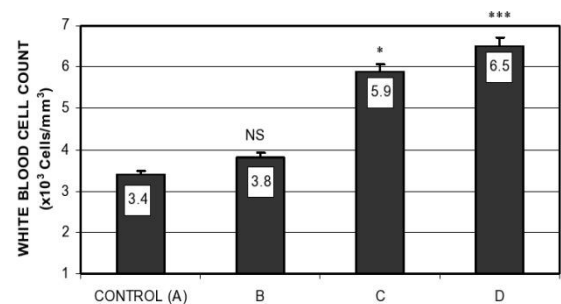
The bleeding time values indicated no significant differences ( $P > 0.05$ ) between the experimental groups: (B,  $2.0 \pm 0.14$  min; C,  $2.0 \pm 0.23$  min; and D,  $1.8 \pm 0.09$  min) and the control group A ( $1.7 \pm 0.13$  min) (Fig.6).



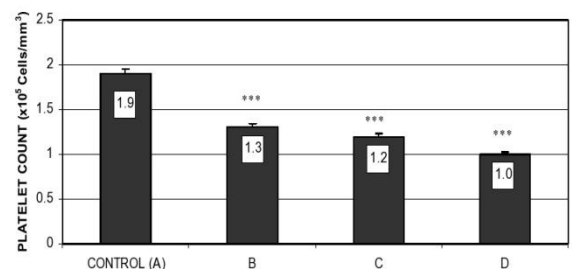
**Figure 1.** The effects of exposure to sub-lethal doses of tobacco snuff concentration on hematocrit. NS; no significance between control and experimental groups.



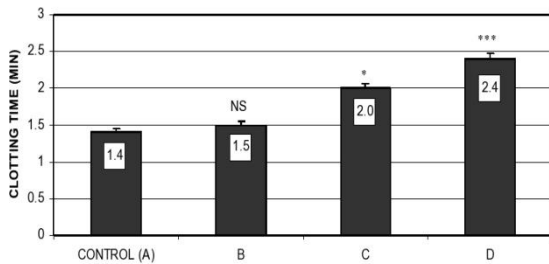
**Figure 2.** The effects of exposure to sub-lethal doses of tobacco snuff concentration on RBC count. NS; no significance between control and experimental groups. \* $P < 0.05$  vs. control group.



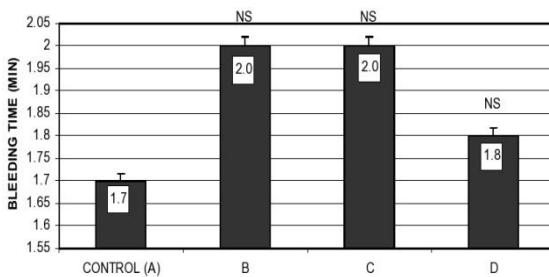
**Figure 3.** The effects of exposure to sub-lethal doses of tobacco snuff concentration on WBC count. NS; no significance between control and experimental groups. \* $P < 0.05$  and \*\*\* $P < 0.001$  vs. control group.



**Figure 4:** The effects of exposure to sub-lethal doses of tobacco snuff concentration on platelet count. \*\*\* $P < 0.001$  vs. control.



**Figure 5.** The effects of exposure to sub-lethal doses of tobacco snuff concentration on clotting time. NS; no significance between control and experimental groups. \* $P < 0.05$  and  $P < 0.001$  vs. control group.



**Figure 6.** The effects of exposure to sub-lethal doses of tobacco snuff concentration on bleeding time. NS; no significance between control and experimental groups.

## Discussion

The present data indicated that at higher sub-lethal doses of tobacco snuff consumption, there was a significant decrease in RBC (8 mg/200 g b.w) and an increase in WBC (6 mg and 8 mg per 200 g b.w) counts of the experimental rats compared to the controls. Previous studies [15, 16] have reported a reduction in RBC count and increase in total WBC count due to consumption of smokeless tobacco in human subjects. Another study by Adeniyi *et al* [11] indicated a reduction in RBC count but no significant effect of tobacco snuff consumption on total WBC count in Wistar rats. Our finding also indicated significant reduction in platelet counts in all sub-lethal doses of snuff consumption. This result concurs with the study by Adeniyi *et al* [11], but contrasts with a similar study which demonstrated an increase in platelet count in Wistar rats administered with tobacco leaf extract compared to their controls [17].

Our data did not show any significant changes in hematocrit between the control group and those administered with doses of tobacco snuff. Scientific literatures suggest that users of smokeless tobacco are similar to non-tobacco users in terms of levels of hematocrit [18, 19]. In contrast, a decrease in

hematocrit has been previously observed in Wistar rats after administering sub-lethal doses of tobacco leaf extracts [11]. Furthermore, we observed significantly more prolonged clotting time in rats administered with higher tobacco dosage compared to controls.

There were no changes in bleeding time at all doses of the experimental groups. We could not find studies demonstrating the effect of tobacco snuff consumption on either blood clotting time or bleeding time. However, a previous study has shown that cutaneous bleeding time was shortened in individuals who smoke high nicotine cigarettes compared to those who smoke nicotine free cigarettes [20]. The above study suggests that cutaneous bleeding time may be accelerated with increase in consumption of nicotine-containing substances, thus contrasting the present finding, which indicated no changes in bleeding time. Another study [21] also demonstrated that blood-clotting time was accelerated by decreasing the nicotine concentration, an alkaloid of tobacco, in a clotting mixture. This finding suggests that blood-clotting time may be prolonged due to increase in consumption of nicotine-containing substances as observed in the present study.

The mechanisms behind the observed effects of tobacco snuff consumption on RBC count, WBC count, platelet count and clotting time are not well understood. However, it has been reported that the invasion of the RBC membrane by peroxidants, which occurs, with the consumption of oxidative drugs can lead to RBC hemolysis [22]. Cotinine, a peroxidant and a metabolite of nicotine found in tobacco products, has also been shown to increase RBC hemolysis in tobacco users at the highest concentration used [23]. This may explain the reduced RBC count observed in the present study at the highest dose of tobacco snuff consumption, which may result to anemia if the animal is exposed to high dosage for a longer period. However, the lack of difference in hematocrit between the control group and the group D rats administered with the highest snuff dosage is in contrast with the observed reduction in RBC count in that group and shows that the hematocrit was not affected by the reduction in RBC count at that concentration.

Tobacco use is associated with an increase in the white blood cell count. This association has been attributed to bronchopulmonary inflammation and/or infection [24]. Increased WBC counts in the present study may suggest chronic inflammatory changes in various tissues, due to exposure to toxic substances in tobacco snuff. Furthermore, the blood

of tobacco smokers and snuffers is reported to contain significant concentration of nicotine [25]. Nicotine is known to cause the release of adrenalin and increases leukocytes in the peripheral blood, bone marrow and spleen [24]. The increase in WBC count in this study may also be attributed to increase in nicotine at higher doses of tobacco snuff, and subsequent release of adrenalin which mobilizes the leucocytes margined in the blood capillaries.

The reduced platelet count observed in this study may be due to platelet aggregation induced by increase in adrenaline release caused by the presence of nicotine in tobacco snuff. A potentiating effect of adrenalin on platelet aggregation has previously been demonstrated [26]. Holmes also demonstrated reductions in circulating platelet count induced by sub-maximal doses of adenosine diphosphate (ADP) which are potentiated by the infusion of adrenaline in rabbits [27]. Abnormal reduction in platelet count will result in thrombocytopenia, affect vascular integrity, and cause increased bleeding and bruising in various tissues. However, the lack of difference observed in bleeding time between the control and experimental animals in this study is an indication that the tobacco snuff-induced reduction in platelet count could not potentiate changes in the bleeding time of the experimental animals.

Furthermore, it has been established that nicotine affects the clot-formation property of the enzyme, thrombin, on the substrate, plasma or fibrinogen (thrombin time) [21]. In that study, higher concentrations of nicotine retarded the clot-formation property of thrombin, thus prolonging the clotting time. This may explain the significantly higher (more prolonged) clotting time observed in the experimental groups administered with snuff compared to the control in the present study. Prolonged blood clotting time results in spontaneous and recurrent bleeding in traumatized or injured body tissues and organs.

In conclusion, the present data indicated that consumption of tobacco snuff at higher sub-lethal doses in Wistar rats reduced RBC count, increased WBC counts, and reduced platelet count. Our findings therefore suggest that the determined effects of tobacco snuff consumption on blood parameters of Wistar rats could be suitably used to extrapolate the effects of chronic tobacco consumption on the physiology of man under sub-lethal condition. These effects may put the body at risk of adverse health conditions such as anemia, inflammatory conditions, thrombocytopenia, reduced vascular integrity, infections and clotting disorders.

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