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Original Article

Effects of Tobacco Smoking on Innate Immunity: A Study Based on Neutrophil Phagocytic Index

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Abstract

The present study was undertaken to find out the effects of tobacco smoking on innate immune mechanism of the body. A total of 60 adult consenting men in the age group of 30 to 50 years were recruited of which 30 were chronic smokers and the rest were non smoking controls. 5ml of venous blood was drawn from each of the subjects and the following parameters were assessed: phagocytic index of neutrophils (which is an index of neutrophil function and is defined as number of neutrophils positive for ingested microbes per 100 neutrophils), total leucocyte count (TLC), differential count of neutrophils. The values from smokers were compared with those from non-smokers. There was a statistically significant decrease in the phagocytic index among smokers when compared to non-smokers (9.44±6.62 vs 28.16±7.31; p<0.0011). An increase in TLC and neutrophil percentage was found in smokers which were not statistically significant (p=0.37 and p=0.12 respectively). Hence it can be concluded that tobacco smoking adversely affects the capacity of neutrophils to ingest microbes and so has suppressive effect on the innate immune mechanism.

Introduction

The tobacco use, either in the form of smoking or smokeless tobacco is the leading preventable cause of death worldwide with more than 5 million deaths per year. As per an estimate, by 2030, the tobacco attributable death is expected to reach 8 million per year (1). A recent study from India estimated that tobacco smoking accounts for about 930000 deaths annually and one in ten adult deaths is related to smoking (2).

It is well known that the smokers are susceptible to a plethora of diseases and conditions like stroke, vascular diseases including myocardial infarction, chronic obstructive pulmonary disease (COPD), multiple cancers, hypertension and osteoporosis. Further, a less recognized fact is that, the smokers

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are more susceptible to various infections (3). There is a growing evidence to suggest that the tobacco smoking might impair the immune system and increase susceptibility to infections (4–6). One of the methods to assess the innate immunity in vitro is calculating the phagocytic index of neutrophils. The phagocytic index is defined as the number of neutrophils with ingested microbes per hundred neutrophils (7). This is calculated by treating one's blood with the candida suspension in a suitable environment and counting the number of neutrophils with ingested candida. With this background, a study was proposed to find out the effect of smoking on the innate immunity which predisposes the smokers to infections.

Material and Methods

This was a study planned to assess the effect of smoking on innate immunity. Following institutional research committee and ethical committee approvals, a total of 60 willing adult men of the age group 30 to 50 years with 30 men being chronic smokers (defined as those with a history of minimum 20 pack years of smoking, study group) and other 30 being never smokers (controls) were recruited. Sample size calculation was performed using OpenEpi version 3 online sample size calculator with prevalence of smokers 30%, power 80% and 95% confidence levels. The minimum sample size required was 58 (29 cases and 29 controls) (8).

Inclusion & exclusion criteria

Study Group (Smokers group) : This group consisted of 30 willing men in the age group 30 to 50 years with a history of minimum 20 pack years of cigarette or beedi smoking (9). Pack year is equal to ([number of cigarettes or beedi smoked per day X number of years] ÷20) (10). To confirm the smoking status, serum cotinine levels were measured using the Qualisa ELISA kits and a value of more than 12.5 ng/ml was taken as the cut off (11). Those men who were on antibiotics, anti-hypertensive drugs or suffered from any ailment at the time of study including diabetes mellitus, COPD and hypertension were excluded. A detailed history regarding current smoking status, number of cigarettes smoked per day and years of smoking was obtained by using a pre-tested questionnaire.

Control group (non-smokers group) : This group included willing men of 30 to 50 years of age with no history of past or present tobacco smoking. The non-smoking status was confirmed by serum cotinine levels (<12.5 ng/ml) (10). Similar to the study group, those who were taking antibiotics, anti-hypertensive drugs or suffered from any ailment including diabetes mellitus, COPD and hypertension at the time of study were excluded.

Collection of the blood sample

Under strict aseptic conditions, 5 ml of peripheral venous blood was drawn from the antecubital vein of the subjects, collected in heparinized sterile bottles and transferred to sterile test tubes immediately for evaluation. The serum cotinine, TLC, differential count (DC) of neutrophils and phagocytic index of the neutrophils were measured on each of the samples drawn. The TLC, using automated analyser (Mindray BC 5300 5 parts differential analyser) and DC, using Leishman's stain technique were estimated (12).

Phagocytic index (7) : The neutrophils in the peripheral blood ingest microbes, when optimal conditions are provided in a medium. The number of neutrophils positive for ingested microbes per 100 neutrophils gives the 'Phagocytic Index'. After half an hour of incubation at 37°C in a suitable medium, normal neutrophil may contain anywhere from 0 to 4 microbes/cell.

Reagents required for estimating phagocytic index

Heat killed Candida suspension, Pooled sera of AB blood group of different healthy individuals, Phosphate buffer saline (PBS), Hanks medium and Lieshman's stain.

Preparation of heat killed microbial suspension

Candida was grown in Sabouraud's 2% dextrose medium for 48 hours at 37°C to obtain organisms in the yeast form. These colonies were then mixed with the PBS using a sterile loop. This mixture

was boiled for 15 minutes and then centrifuged at 3000 rpm for 10 minutes. The deposits were washed with 5 ml PBS and stored at 4°C. This heat killed microbial suspension was counted in improved Neubauer's chamber and optimum amount of the suspension required for the procedure was standardized.

Procedure for estimating phagocytic index

The heparinised blood sample was centrifuged at 2500 rpm for 10 minutes and plasma was discarded. The buffy coat was aspirated carefully and transferred to another test tube. 200 µl of pooled sera, 100 µl of candida suspension and 200 µl of Hank's medium were added to the buffy coat preparation from the test subject. The tube was kept in a water bath for incubation at 37°C for 30 minutes. The test tube was centrifuged at 1500 rpm for 5-10 minutes. The clear supernatant solution was discarded and the buffy coat was aspirated, taken on glass slides and smears were prepared.

Staining

The smears were stained with the Leishman's stain and examined under oil immersion for the presence of microbes inside the neutrophils (Fig. 1). The number of neutrophils positive for ingested microbes per 100 neutrophils was recorded as the 'Phagocytic Index' (13).

Statistical analysis

The data was recorded on a predesigned proforma and managed using MS-Excel 2007 (Microsoft Corporation, Redmond, WA). The descriptive statistics such as mean and standard deviation were calculated. The unpaired student's *t*-test was performed to compare the means between cases and controls. A p-valued ≤0.05 was considered significant. All statistical analysis were performed with the help of SPSS (Statistical Package for Social Sciences) version 20.0 (IBM Corp., Armonk, NY).

Results

The mean age of the study group was 39.2 (±8.2) years, mean height was 164.7 (±7.23) cm and the mean weight was 55.76 (±9.5) kilograms. Among the smokers, majority smoked cigarettes (86.7%), and 50% were light, 26.7% were moderate and 23.3% were heavy smokers. All smokers had serum cotinine

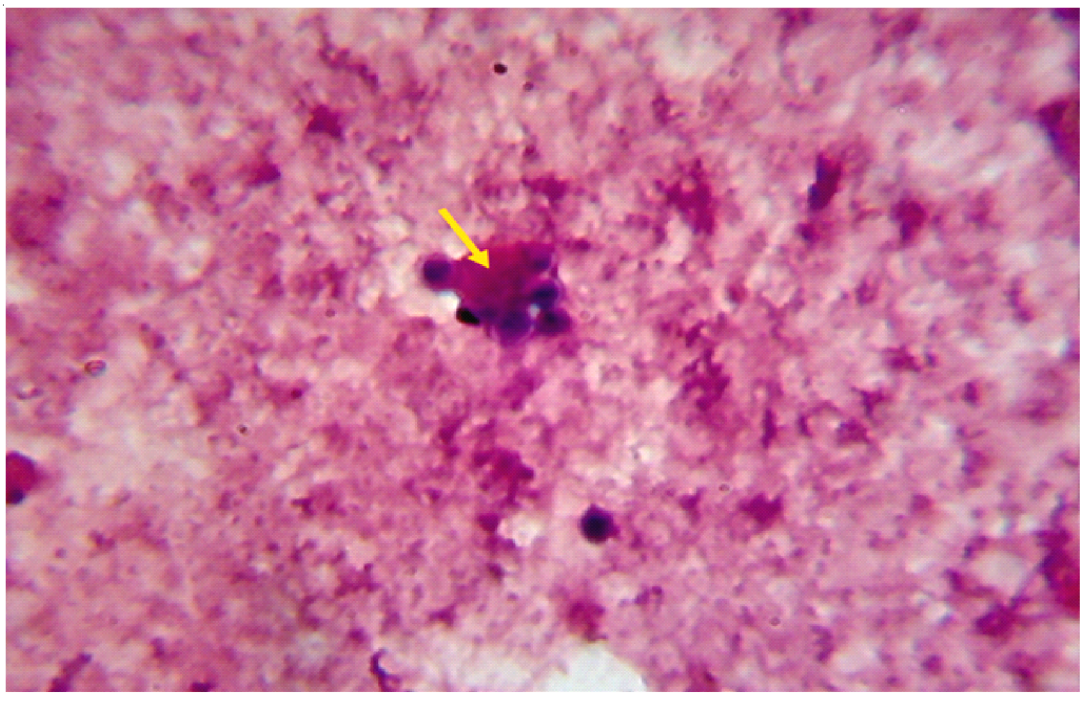

Fig. 1: Polymorphonuclear cell with ingested Candida in chronic smoker (arrow mark, x 100).

levels > 12.5 ng/ml and non-smokers had serum cotinine levels less than 12.5 ng/ml. There was a statistically significant decrease in the phagocytic index in the study group (smokers) when compared to the control group (non-smokers) [9.44±6.62 vs 28.16±7.31; p<0.0001] (Fig. 2). An increase in TLC [8261.29±2728.57 vs 7677.41±1676.24] and DC of neutrophils [58.26±8.33 vs 55.26±8.48] were also observed in the smokers when compared to non-smokers, but these were not statistically significant [p=0.37 and p=0.12 respectively].

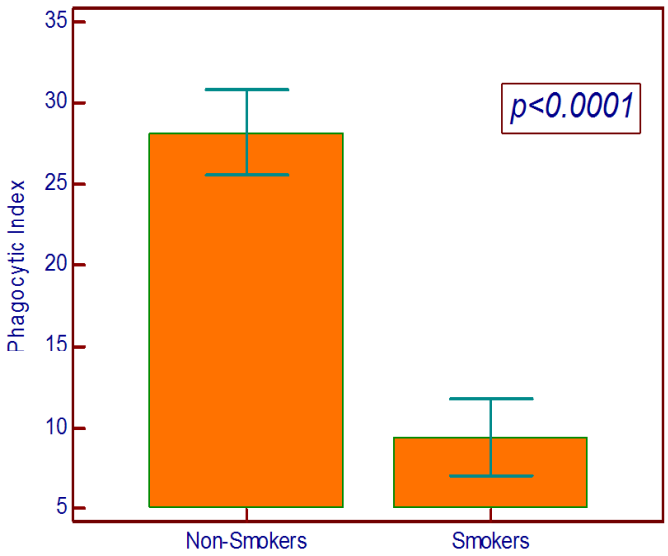

Fig. 2 : Mean±SD (n=30/group) Phagocytic index of PMN from non-smokers vs smokers.

Discussion

Tobacco smoking affects both the innate and adaptive immunities and this in turn possibly predisposes the smokers to a number of infectious diseases (14, 15). The tobacco smoke consists of several toxic and carcinogenic substances such as tar, nicotine, ammonia, carbon monoxide, carbon dioxide, acrolein, formaldehyde, hydroxyquinone, acetone and cadmium (16). These substances have harmful effects on human health such as increased susceptibility to respiratory infections, cardiovascular diseases including myocardial ischaemia, stroke, COPD and lung cancer (17). Tobacco smoke affects the functioning of white blood cells and hence has got an immunosuppressive effect (18, 19). Some earlier studies have suggested that PMNs in smokers exhibited depressed migration and chemotaxis when compared to non-smokers (20–22). Nicotine is one of the key ingredients of tobacco smoke which has a dose and duration dependent toxic effect on immune system (18). However the global influence of tobacco smoke on immunity is still not clear (23).

The present study was conducted to assess the impact of smoking on innate immune mechanism in a sample of apparently healthy chronic smokers. Neutrophils are an important component of innate immunity. Whenever neutrophil functioning or number is decreased, an increased susceptibility to infections is observed (24). The study showed that the phagocytic function of neutrophils was adversely affected by the tobacco smoking. This is in accordance with studies by Guntsch A et al and M Srinivas et al (4, 5). The process of phagocytosis involves various steps like margination, diapedesis, chemotaxis, opsonization, engulfment, degranulation and finally killing phase (25). Further research could throw light on which of these steps are affected by the tobacco smoke.

In the present study, increase in TLC and DC of neutrophils were found which were not statistically significant. The possible reasons could be inflammation of bronchioles, chronic tissue damage and nicotine induced catecholamine release (26, 27).

Infectious diseases are one of the major causes of morbidity and mortality from smoking along with cancer, heart disease and chronic lung disease. The findings emphasize the need to include smoking cessation as a part of preventive and therapeutic plan for smokers suffering from serious infections. These findings could be extrapolated to environmental exposure to tobacco smoke, so called second hand smoking or passive smoking among household contacts of an active smoker, who are at an increased risk of developing infections (28).

The findings also stress the need to control second hand tobacco smoke exposure (passive smoking) for prevention of infectious diseases especially among individuals who are constantly exposed to active smokers.

Conclusions

The present study confirms that in chronic smokers, the phagocytic ability of the PMNs is actually reduced. A larger study may be advisable to confirm the findings.

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