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Assessment of Genotoxicity in Lymphocytes of Active and Passive Cigarette Smokers Attenuated with Green Tea

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ABSTRACT

The well-reported carcinogens in tobacco smoke are polycyclic aromatic hydrocarbons and nitrosamines cause the production of DNA adducts and leading to lung cancer. However, the adverse effects of cigarette smoke on other tissues are largely unknown. We explored the genotoxic effects of tobacco smoke on peripheral lymphocytes of active and passive smokers. Blood samples were isolated from 100 males' including 25 active-, 25 passive- and 50 non-smokers. Alkaline comet assay was done and classes were defined on the basis of comet tail length. Significant differences were found in total comet score (TCS) among different groups (p<0.05). The TCS value of active smokers were significantly increased with other narcotics like charas and snuff (p<0.05). The passive smokers using snuff were having significantly higher TCS values then the control (p<0.05). Reduction in TCS values of active smokers using green tea on daily basis were statistically significant (p<0.05). Taking together, our results indicate that tobacco smoking highly induces DNA damage in blood cells. Additionally, green tea significantly reduces the toxic effects of smoke which is a hope to rescue tissue exposed to smoke toxins.

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Authors' Contribution
MK developed the idea and supervised the project. SG and AA performed the experiments. Attaullah and SG collected the sample. GNK analyzed the data and wrote the manuscript.

Key words
Cigarette smoking, Genotoxicity, Comet assay, Total comet score, Green tea.

INTRODUCTION

Tobacco smoke contains a variety of tobacco-specific carcinogens obtained from nicotine formed during tobacco processing (Shakoori *et al*., 2018; Hecht, 1999). Common examples of these derivatives are 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N′-nitrosonornicotine (NNN) (Schuller and Orloff, 1998). The mutagenic and tumor-promoting activities of nicotine may result from its ability to damage the genome, disrupt cellular metabolic processes, facilitate growth and spreading of transformed cells (Grando, 2014). Some types of cancers related to nicotine are small-cell and non-small-cell lung carcinomas, as well as head and neck, gastric, pancreatic, gallbladder, liver, colon, breast, cervical, urinary bladder and kidney cancers (Aveyard *et al*., 2002). According to previous reports, the nicotinic acetylcholine receptors (nAChRs), which are triggered by nicotine, can activate several signaling pathways that can have tumorigenic effects (Grando, 2014). However, it stimulates dopamine release which causes pleasure and increases the working ability for a short time (Pontieri *et al*., 1996). Green tea (*Camellia sinensis*) derived beverages are commonly used in daily life throughout the world. The general chemical composition of these beverages is polyphenols (flavanols and flavonols) and catechin, epigallocatechin-3-gallate (EGCG) (Chacko *et al*., 2010). According to previous studies, the whole extract of green tea or EGCG helps in the prevention of various types of malignancies including breast cancer (Koo and Cho, 2004; Lin and Lin-Shiau, 2006). Majority of carcinogenic substances like nicotine induces DNA damage which promotes genomic instability leading to cancer. There are many well-developed methods to assess DNA damage such as Comet assay to measure the effect of different chemicals on DNA or at the genetic level that causes cancer (Singh *et al*., 1988; Collins, 2004). In this study, we aimed to evaluate the genotoxic effect of tobacco smoke in its addicts (active smoker). Furthermore, we tried to find out the genotoxic effect of environmental tobacco smoke in the lymphocytes of active smoker's relative (passive smoker). Importantly, we evaluated the effects of green tea against tobacco smoke-induced genotoxicity in

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human lymphocytes.

MATERIALS AND METHODS

Blood sampling

Blood samples were collected from the 100 males, Non-smokers (50%), cigarette smokers (25%) and their close friends or relatives (25%) belong to Peshawar city of Khyber Pakhtunkhwa province, Pakistan. Complete history regarding the participants was collected via a questionnaire, including information about age, occupation, duration of usage, daily consumption and health problems. The questionnaire was also given to the control group with modifications like the omission of the question related to the duration of usage and their answers were also recorded.

Isolation of lymphocytes from the blood

Mixture (1:1, 3 ml) of EDTA containing blood and phosphate buffer saline (PBS, Ca^{++}, Mg^{++} free) was layered over 2 ml ficoll / lymphocytes separation medium (LSM-1077; Catalog Number: HiSep LSM™ 1077-LS001) in a 15 ml falcon tube. The mixture was centrifuged at 2000 RPM for 30 min. Four distinct layers; the plasma layer (upper most layer), buffy coat / lymphocyte and monocyte layer (the second layer), ficoll layer / LSM (third layer), RBCs and other cell debris (bottom) were seen after centrifugation. The buffy coat was isolated with glass Pasteur pipette without interrupting other layers, mixed with 1 ml PBS and centrifuged at 1500 RPM for 10 min. The pellets were mixed with 1 ml PBS again and centrifuged at 1000 RPM for 10 min. After a wash with PBS, the cell pellets (lymphocytes) were gently mixed in 1 ml PBS and used for the different set of experiments.

Experimental design

Fifty non-smokers healthy individuals were kept as control. Twenty Five individuals who were addicted to cigarette smoking were named active smokers. Twenty Five males who did not take cigarette smoke but were found very close to active smokers were named passive smokers.

Comet assay

Comet assay, a single cell gel electrophoresis assay of the blood samples was carried out manually at our laboratory with slight modifications, established by Singh *et al*. (1988).

After keeping in 100% ethanol for 20 min, the sample containing slides were fully hydrated for 30 min in distilled water (DW) followed by staining with Acridine Orange (20 µg/ml) for 5 min. Finally, the slides were washed with cold DW and mounted with the cover glass.

For scoring of DNA comets, 100 stained nuclei were selected randomly from each group under the fluorescent microscope at 200x magnification and images were recorded. For total comet score (TCS), five classes ranging from class 0 (having no nuclear damage and hence no tail), Comet Class 1 (comet nucleus diameter 1.5 times less than tail), Comet Class 2 (comet nucleus diameter less than1.5-2.0 times the tail), Class 3 (comet nucleus diameter 2.0-2.5 times less than the tail) and Class 4 (maximum damage with almost total DNA in tail). The total comet score was calculated by formula; TCS=0(n) + 1(n) + 2(n) + 3(n) + 4(n), where (n) shows number of cells in each class.

Statistical analysis

Statistical analyses were performed using SPSS software (v.12.0). Differences among experimental groups were calculated by one-way ANOVA, while Student's t-test was used to analyze the statistical difference between groups. The p-value <0.05 was taken for the statistical significance.

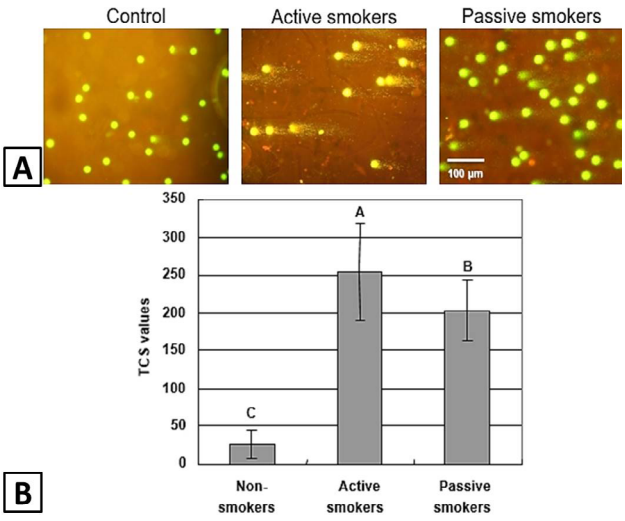

Fig. 1. Analysis of lymphocytes-derived DNA comets in different groups. **A**, lymphocytes nuclei of control (non-smokers), active- and passive smokers were analyzed for DNA damage by comet assay. Control group has intact nuclei with no tail. In case of active smokers group, the comets of significant length were observed compared to control. Also, the nuclear damage was found in passive smoker group. However, it was less than active smoker group. **B**, quantitative analysis of TCS values among different groups of active and passive smokers compared to control group. A, B and C are the Duncan's variables representing statistical significance among groups. Quantitative analysis showed a significant increase in DNA damage in active and passive smokers compared to control group (*p<0.05).

RESULTS

Analysis of lymphocytes-derived DNA damage in different groups' of smokers

To evaluate the effect of tobacco smoke on blood-lymphocytes DNA damage of active- and passive smokers, comet assay was conducted. A remarkable DNA damage frequency was observed in active smokers compared to the control group. Mean TCS of active-, passive and control group was found 253.25±64.04, 203.16±38.98 and 25.84±18.63, respectively. Control group has intact nuclei with no tail. In case of active smokers, the comets of significant length were observed compared to control. Also, the nuclear damage was found in the passive smoker group. However, it was less than the active smoker group (Fig. 1A). The quantitative analysis of TCS values among different groups showed a significant increase in DNA damage in active and passive smokers compared to the control group (Fig. 1B; $p<0.05$).

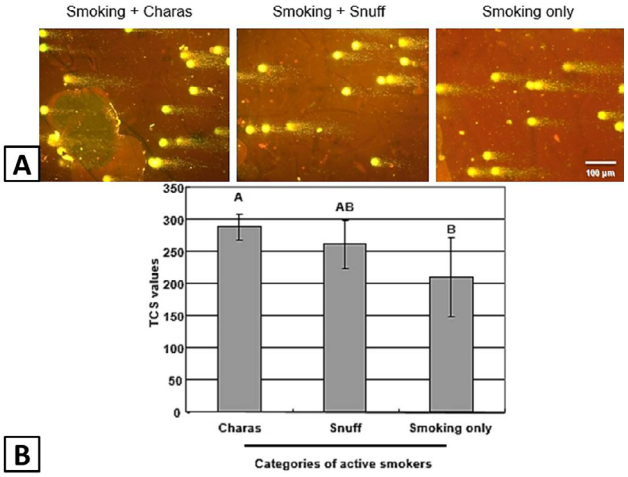

Fig. 2. Measurement of DNA damage in Snuff and Charas-addicted active smokers. **A**, comparison of DNA damage by showing comets among different groups of active smokers. In addition to smoking, DNA damage was significantly increased with consumption of charas and snuff. Compared to active smokers, the length of the comet was found longer in charas and snuff-addicted active smokers. **B**, quantitative analysis among charas and snuff-addicted smokers. A, B and C are the Duncan's variables representing statistical significance among groups. Quantitative analysis showed that charas-addicted active smokers have highest TCS value among all three groups. However, the TCS values of snuff-addicted people were also higher than those who did smoking only (*$p<0.05$).

Evaluation of DNA damage in snuff and charas addicted active smokers

Next, we assessed the DNA damage in those active smokers population who were addicted to charas (also called hashish or hash, a handmade product derived from the cannabis plant) and snuff (the product which is made of ground tobacco leaves, a small amount of calcium carbonate and a tree bark ash). DNA damage was significantly increased with consumption of charas and snuff. Compared to active smokers, the length of the comet was found longer in charas and snuff-addicted smokers (Fig. 2A). Quantitative analysis showed that charas-addicted smokers had the highest TCS value among all three groups. However, the TSC values of snuff-addicted people were also higher than those of active smokers only (Fig. 2B; $p<0.05$).

Analysis of DNA damage in different groups of passive smokers

In continuation to charas and snuff addicted active smokers, next, we evaluated the levels of DNA damage in snuff-addicted passive smokers. The level of DNA damage was found higher in snuff-addicted passive smokers compared to passive smokers who were only exposed to cigarette smoke (secondary smokers) (Fig. 3A). Quantitative analysis showed a significant increase in TCS values of snuff-addicted passive smoker compared to those who were not using snuff (Fig. 3B; $p<0.05$).

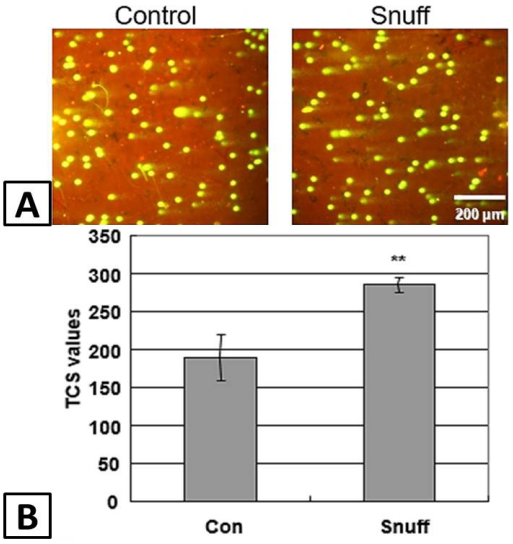

Fig. 3. Measurement of DNA damage in different groups of passive smokers. **A**, DNA damage was evaluated in passive smokers who were addicted to snuff. Compared to passive smokers, the level of DNA damage was found higher in those who were addicted to snuff too. **B**, quantitative analysis of TCS values between passive smokers and those who were addicted to snuff. A significant increase in TCS values was found in snuff-addicted passive smoker compared to those who were not using snuff (**$p<0.005$).

Estimation of DNA damage in different classes of active smokers addicted to charas and snuff

We further quantify the levels of DNA damage in five different classes of active smokers who were using charas and snuff too. The numbers of cell's nuclei which satisfied the criteria for class 0 were very few in all three groups of active smokers. The total TCS values were gradually increased in different classes of active smokers. In case of active smokers with no other addiction, the level of DNA damage of a maximum number of nuclei belonged to class 2. Compared to only smoking- and snuff-addicted groups of the smoker, the highest DNA damage was found in a charas-addicted group. The nuclei belonged to class 4 *i.e.* maximum damage with distorted nuclei, were found in charas-addicted smokers. However, the lowest TCS values were found in class 4 of non-addicted smokers (Fig. 4).

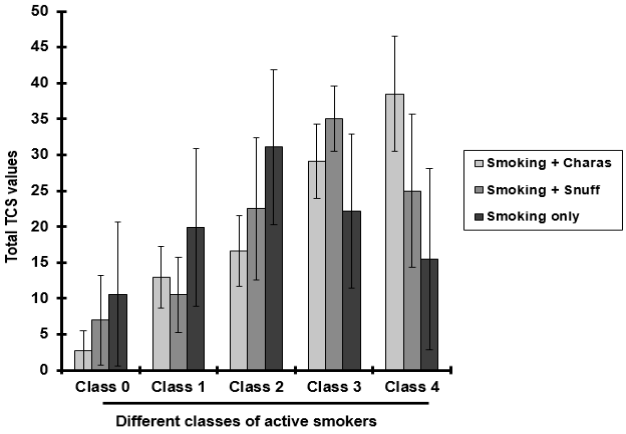

Fig. 4. Evaluation of DNA damage in different classes of active smokers addicted to Charas and snuff. Quantification analysis of the levels of DNA damage in five different classes of active smokers addicted to charas and snuff. The numbers of cell's nuclei which satisfied the criteria for class 0 were very few in all three groups of active smokers. The total TCS values were gradually increased in different classes of active smokers. The highest number of distorted nuclei with maximum DNA damage was found in charas-addicted smokers. However, this number was low in active smokers who were not addicted to charas or snuff.

Attenuation of DNA damage with green tea consumption in active smokers

Finally, we evaluated the effects of green tea against tobacco-induced DNA damage in human blood lymphocytes. The length of DNA comets was greatly reduced in active smokers who were using one or two cups of green tea every day (Fig. 5A). Quantitative analysis showed a slight improvement in nuclei morphology in class 0. No significant differences were found with green tea in classes, 1, 2 and 3, respectively. The TCS values in class 4 of active smoker using green tea (13.76±14.05) were reduced 2.5 fold compared to active smokers (35.83±11.28) with no usage of green tea on daily basis (Fig. 5B). In addition, the total TCS value of green tea using active smokers (213.85±61.58) was largely reduced compared to active smokers (274.83±36.02; Table I).

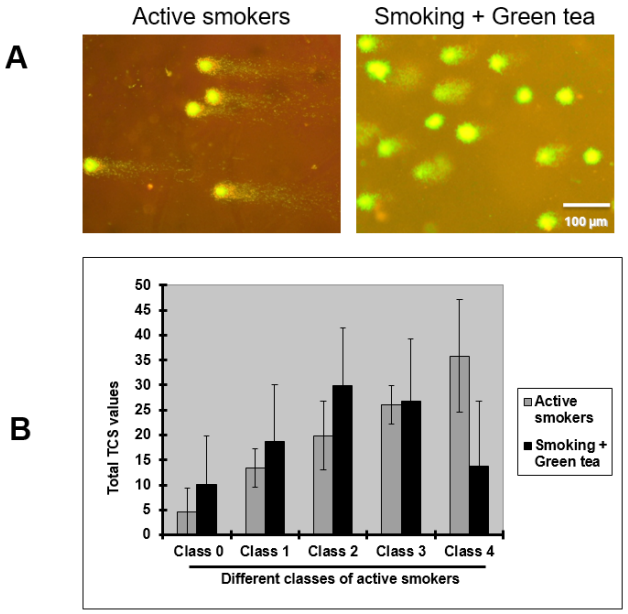

Fig. 5. Attenuation of DNA damage with green tea consumption in active smokers. **A**, comparison between active smokers not taking- and those who were using one or two cups of green tea every day. The length of DNA comet was greatly decreased in active smokers who were using green tea. **B**, quantitative analysis of DNA damage in different levels of active smokers and those who were using green tea. The analysis showed that green tea significantly reduced the TCS values or DNA damage in class 4 compared to active smokers with no usage of green tea on daily basis.

Table I.- Evaluation of TCS values on the basis of green tea usage among active smokers.

Comet classes	Green tea	
	Yes (n=12)	No (n=13)
0	4.67±4.92	10.15±10.12
1	13.42±4.08	18.77±11.88
2	19.92±7.19	29.85±12.04
3	26.08±4.08	26.77±13.02
4	13.77±14.62	35.83±11.78
Total TCS values (Mean ± SD)	213.85±61.58	274.83±36.02

DISCUSSION

In this study, we disclosed the effects of cigarette smoke on DNA damage of blood lymphocytes in active and passive smokers. Also, we found out significant increase in the genotoxic effects of tobacco smoking with additional narcotics *i.e.* charas and snuff. Interestingly, the level of DNA damage was greatly recovered with daily consumption of green tea in active smokers. So far, the toxic effects of tobacco smoking have been reported in different organs, especially, lungs (Aveyard *et al*., 2002). However, the direct genotoxic link of tobacco smoke with blood lymphocytes is not very clear. Therefore, we designed the study to find out the possible genotoxicity of tobacco smoke-derived toxins on blood lymphocytes and the protective role of green tea against it by using comet assay.

Comet assay is a simple and reliable approach to measure and quantify the level of genotoxicity of a toxin, mutagen or carcinogen. There are some limitations to measure the exact length of comet or quantification of comets intensity. Although various software packages are being used nowadays, which works based on the above principles. However, that is quite expensive and cannot be afforded by the low grant research groups. A new visual method of scoring was introduced based on the degree of DNA damage and appearance of the comet tail (Collins, 2004). The same method was adopted here to measure the level of tobacco smoke-induced DNA damage. Including active smokers, passive smokers were also affected by tobacco smoke, which clearly reflects the negative effects of interaction with habitual smokers in society. Furthermore, the consumption of narcotics like charas by active smokers and snuff in both groups were found extremely hazardous for health and induces genotoxicity.

The pharmacological effects of green tea are reported in many studies (Koo and Cho, 2004; Cabrera *et al*., 2006; Syed *et al*., 2007; Chacko *et al*., 2010). We showed that green tea has the potential to repair or inhibit the smoke toxin-induced DNA damage. Although we did not evaluate the activities of individual bioactive components of green tea. However, the previous studies have reported the antioxidant activities of green tea-derived compounds (Chacko *et al*., 2010). The EGCG, a major bioactive component of green tea suppress inflammation, proliferation, and angiogenesis induced by cigarette smoke in normal human bronchial epithelial cells via inactivation of NF-κB signaling (Syed *et al*., 2007). Therefore it is concluded that reduction in the DNA damage of lymphocytes of active smokers may be due to the bioactivity of EGCG and other compounds in green tea.

Altogether, our results showed that tobacco smoking significantly induces DNA damage in lymphocytes of active and passive smokers. The intensity of DNA damage significantly enhances with additional consumption of narcotics like charas and snuff in both groups of smokers. The passive smokers using snuff are having the higher risk of their DNA damage compared to control. Consumption of daily usage of green tea significantly reduce or attenuate the effects of tobacco smoke-derived toxins. This study shows the adverse effects of cigarette smoke at the genetic/DNA level. Furthermore, it reflects the importance of green tea-derived beverages as a source to neutralize the effects of toxins to which our body expose in daily life.

Statement of conflict of interest

No conflict of interest is there to disclose

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