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Regular black tea habit could reduce tobacco associated ROS generation and DNA damage in oral mucosa of normal population

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ABSTRACT

Tobacco and tea habit are very common in world wide. In the present study, an attempt was made to evaluate the effect of regular drinking of black tea on reactive oxygen species (ROS) generation and DNA damage in buccal cells of normal subjects with or without tobacco habit. Expression of ROS associated proteins IκB, NF-κB as well as DNA repair associated proteins p53, MLH1 were also analyzed. Exfoliated buccal cells were collected from 308 healthy individuals and classified according to age, tobacco and tea habits. In all age groups, comparatively high ROS level and significantly high DNA damage frequency were seen in individuals with tobacco habit than the subjects without tea and tobacco habits. Tea habit effectively lowered ROS level and restrict DNA damage in tobacco users irrespective of ages. The DNA damage seen in the subjects was not associated with apoptosis. Moreover, tea habit effectively lowered the expression of IκB, NF-κB, p53 and MLH1 in tobacco users in all age groups. It seems that regular black tea habit could have anti-genotoxic effect as revealed by reduced tobacco associated ROS generation and DNA damage in buccal cells.

1. Introduction

Epidemiological studies have demonstrated the association of regular tobacco use with multiple health ailments including cardiovascular disease, chronic lung disease and cancer especially in lung, head and neck, kidney etc., (Hart et al., 2010). Chronic tobacco habit can lead to development of oxidative and genotoxic stress particularly in the buccal epithelium as it is the primary exposure site for tobacco associated carcinogens (Csiszar et al., 2009). Imbalance between ROS generation and antioxidative defence mechanism by enzymatic and non-enzymatic (IκB/NF-κB) procedures resulted development of oxidative stress. It was reported that activation of NF-κB occurs in response to oxidative stress through degradation of IκB and translocation to nucleus for transactivation of different target genes including IκB (Sachdev et al., 1998). NF-κB activation has been reported to be modulated by different antioxidants (Van der Berg et al., 2001). The persistent oxidative stress could generate DNA strand breakage which is sensed by p53, resulting in its activation and cell cycle arrest at G1/S boundary, to repair the DNA or undergo cellular apoptosis (Waris and Ahsan, 2006; Efeyan and Serrano, 2007). In tobacco associated oral cancer, impairment of mismatch repair genes MSH2 (mutS Homolog 2), MLH1 (mutL Homolog 1) leads to high frequency of chromosomal instability (Sengupta et al., 2007).

Different experimental studies suggested that regular tea habit is associated with improved health benefit which is attributed to the antioxidative property of tea polyphenols (Khan and Mukhtar, 2007; Yang et al., 2009). It is possible that regular drinking of tea may modulate the oxidative and genotoxic stress in oral cavity, generated due to chronic tobacco habit. Several epidemiological studies analyzed the effect of both green tea and black tea consumption on plasma or urine antioxidant activity in normal individuals (Han et al., 2010; Hakim et al., 2003). Unfortunately, these studies did not sensitize the effect of regular tea habit on buccal cells of tobacco users, except Chung et al. (2003) who showed that regular intake of green tea could increase apoptosis in buccal cells of smokers. In India, black tea consumption is more popular than green tea. It was reported that tea polyphenols present in black tea could restrict lung carcinogenesis in an experimental mouse model (Manna et al., 2009). However, to the best of our knowledge no study has yet been done in detail to understand the effect of regular black tea drinking on prevention of oxidative and genotoxic stress in buccal cells of tobacco users.

Thus a population based analysis has been undertaken to understand the preventive role of black tea, if any, on tobacco associated ROS generation and DNA damage in exfoliated buccal cells of tobacco users. ROS level and DNA damage were measured in buccal cells of normal individuals. In addition, frequency of

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apoptosis and expression pattern of IκB, NF-κB, p53 and MLH1 were also analyzed to delineate the mechanism of antigenotoxic effect of black tea. Our data show that regular consumption of black tea could effectively reduce the high ROS level and high DNA damage frequency seen in only tobacco users, probably through modulation of anti-oxidative and DNA repair pathways.

2. Materials and methods

2.1. Studied population

A total of 308 healthy individuals participated in this project with informed consent. Studied subjects were screened using a questionnaire to find out the possible factors (age, tobacco habit, tea habit) that could affect ROS generation and DNA damage. All other parameters were taken as constant. General characteristics of studied samples are summarized in Table 1. Different types of tobacco habit such as cigarette (66 subjects), bidi (a native cigarette-like stick of coarse tobacco hand-rolled in a dry tendu leaf of *Diospyrus mebunoxylon* or *Diospyrus ebenum,* 36 subjects) and chewing tobacco (44 subjects) were considered. Subjects having both cigarette and bidi habits, occasional smokers and ex- smokers were not considered for clarification of data (see Supplementary Table 1). Five cigarettes or equivalent amount of tobacco used is considered the average tobacco consumption per day in population. Therefore, subjects who used at least five cigarettes or equivalent amount of bidis or chewable tobacco, per day for at least 1 y were considered as tobacco user. Tobacco content in one cigarette is nearly equal to that present in two bidis and chewing tobacco used 7–8 times per day may be equivalent to the smoking 30–40 cigarette per day (Majumdar et al., 2005; Reddy and Shaik, 2008). So chewing tobacco used once is nearly equal to 5 cigarettes per day or 10 bidis per day. In this study, the duration nearly 25 min per chewing tobacco, 5 min per cigarette and 2–3 min per bidi smoking were considered. In this study, four cups of tea consumption per day was considered as minimum tea habit (Hakim et al., 2003). Subjects who take 4–10 cups of black tea per day for at least 1 y were selected as subjects with tea habit. Occupation of subjects was not considered though they are from different occupations (Table 1).

2.2. Study design

Subjects were classified into two age groups (1) <65 and (2) >65 y. Initially subjects were classified into four age groups i: < 20, ii: 25–40, iii: 45–60 and iv: >65 y. For clarification of data five intermediate years between the age groups were omitted. As first three age groups showed similar pattern of results, they were clubbed together as <65 y age group. Each age group was further classified in four different subgroups on the basis of tea and tobacco habit [−tea/−tobacco (taken as control group); −tea/+tobacco; +tea/−tobacco; +tea/+tobacco] (Table 1). Buccal cells of all individuals were analyzed for ROS generation and DNA damage. Cellular apoptosis was analyzed in 32 randomly selected subjects from different age groups. The expression of IκB, NF-κB were analyzed in 48 subjects while, expression of p53 and MLH1 were analyzed in 60 and 48 subjects respectively, randomly selected from different age groups.

Table 1
Demography of the Subjects.

Factors	Groups	Number of subjects		
		Non-tobacco users	Tobacco users [a]	Total
Total		162	146	308
Age	<65 y	131	118	249
	>65 y	31	28	49
Sex	Male	69	125	194
	Female	93	21	114
Occupation	Student	65	42	107
	Service	23	30	53
	Business	10	26	36
	Daily labour	15	25	40
	Retired	14	22	36
	Housewife	35	1	36
Tea habit [b]	No tea habit	88	68	156
	Subjects with tea habit	74	78	152

[a] Subjects used at least five cigarettes or equivalent amount of bidis or chewable tobacco, per day for at least 1 y were considered as tobacco user.
[b] Subjects take 4–10 cups of tea per day for at least 1 y are considered as subjects with tea habit.

2.3. Sample collection

Exfoliated buccal cells were collected from normal healthy subjects by oral brushing in the morning before taking any tobacco or tea. Prior to brushing subjects were asked to wash their mouth with normal saline (0.9% NaCl solution) to avoid the interference of mucus. Collected samples were taken in phosphate buffer saline (PBS), washed and resuspended in PBS and used for ROS detection and comet assay. Cell smear was prepared for apoptosis and immunocytochemistry (ICC). All the experiments were done with freshly collected samples.

2.4. Measurement of intracellular ROS levels

Cellular ROS level was measured by ROS sensitive dye DCFH-DA (Yamamoto et al., 2003). Cell suspension (100 μl) was incubated with DCFH-DA (10 μM) for 10 min at room temperature in the dark and fluorescence intensity (F.I 1) was measured at λ_{ex} 505 nm and λ_{em} 529 nm using spectrofluorimeter (Varian Cary Eclipse, plant 6000). Then, H_2O_2 (800 μM) was added in the same mixture and again fluorescence intensity (F.I 2) was measured. The ROS level was calculated as: ROS level = 1/[(F.I 2)–(F.I 1)] (Supplementary information). Mean ROS level of each group was compared with mean ROS level of control subjects of respective age groups.

2.5. Evaluation of DNA damage by alkaline single cell gel electrophoresis

DNA damage in the exfoliated buccal cells was assessed by single cell gel electrophoresis (comet assay) by using a standard protocol with some modifications (Szeto et al., 2005). Cell suspension mixed with 1% low melting point agarose in 1:2 ratio and spread on a microscopic slide pre-coated with 1% normal agarose. Slides were then treated with trypsin–EDTA solution (0.25% trypsin, 1 mM EDTA) for 30 min followed by proteinase K (1 mg/ml) digestion for 1 h at 37 °C. Slides were then equilibrated in electrophoresis buffer (10 mM NaOH, 1 mM EDTA, pH: 9.1) for 10 min and electrophoresed at 30 V for 20 min followed by neutralization for 15 min in 400 mM Tris/HCl, pH 7.4. After staining with ethidium bromide (20 μg/ml) DNA damage was visualized under fluorescence microscope (Leica DM 4000 B, Germany) using 515–560 nm excitation filter. Hundred cells from each individual were randomly scored for DNA damage analysis using Komet 5.5 image capture software and analysis system (Kinetic imaging limited, Liverpool, Merseyside, UK). The percent tail DNA (TD%) and Olive tail moment (OTM) were measured by the software for DNA damage analysis. The Mean TD% and OTM for each group were compared with mean values of control subjects of respective age groups.

2.6. Determination of apoptosis rate in the buccal cells by TUNEL assay

Apoptosis analysis was done in the buccal cells using TUNEL assay kit (Roche Molecular Biochemicals, Manheim, Germany) according to manufacturers' protocol. Cell smear was fixed in freshly prepared 4% paraformaldehyde in PBS followed by endogenous peroxidase blocking with 3% H_2O_2 in methanol for 10 min at room temperature. Then the cells were permeabilized with 0.5% triton-X 100 and incubated with TUNEL reaction mixture at 37 °C for 60 min in a humidified chamber. After washing, cells were incubated with horseradish peroxidase conjugated anti-fluorescein antibody at 37 °C for 30 min in a humidified chamber. Stained cells were visualized after substrate (DAB) reaction by light microscope. For each sample six randomly chosen fields were scored in a blinded manner. In each group mean apoptotic cell percentages were compared with mean apoptosis percentage of control subjects of respective age group.

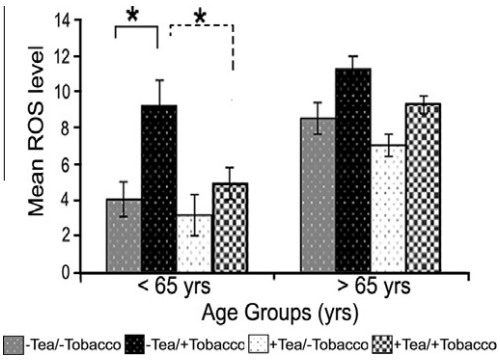

Fig. 1. Pattern of mean ROS level among subjects of <65 and > 65 y. age group; Solid line represents the significant level with respect to control subjects of each age group. Dashed line represents the significant level with respect to only tobacco user subjects. ∗ represent significant level (p value < 0.05).

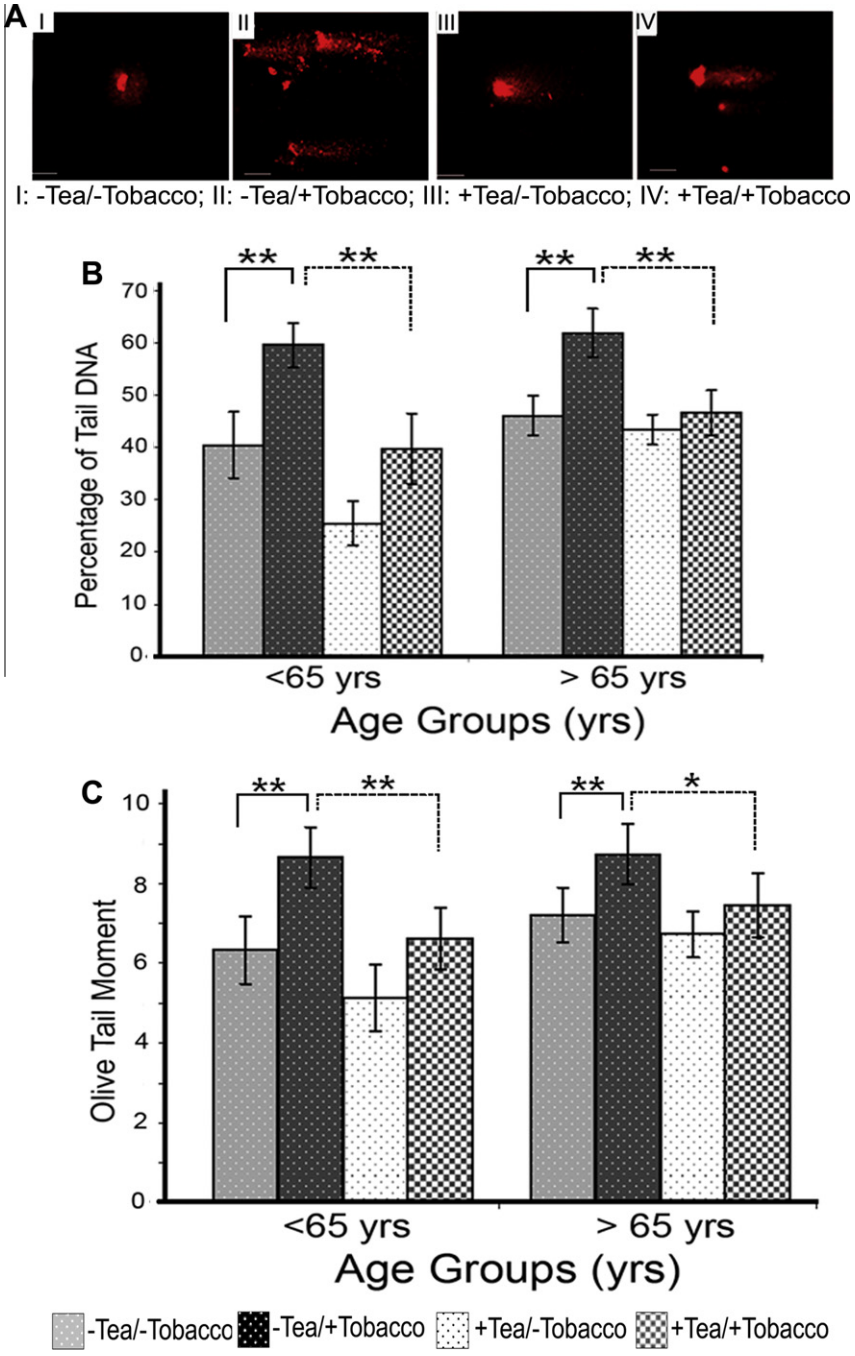

Fig. 2. (A) Representative photographs of DNA damage by alkaline single gel electrophoresis (I–IV) (comet assay) of exfoliated buccal squamous cells. (Magnifications 40×. Scale bar 50 μm). Graphical representation of (B) percent tail DNA and (C) olive tail moment in subjects of <65 y. and >65 y. age groups, with/without tobacco and/or tea habits. Solid line represents the significant level with respect to control subjects of each age group. Dashed line represents the significant level with respect to only tobacco user subjects. ∗ represent significant level (p value < 0.05) and ∗∗ represent significant level (p value < 0.01).

2.7. Immunocytochemical analysis

Exfoliated buccal cell smear was fixed in methanol for 30 min at −20 °C. Cells were permeabilized with 0.5% Triton-X, followed by endogenous peroxidase blocking with 0.5% H_2O_2 in 1X PBS. After that cell smear was blocked with 3% BSA in 1X PBS for 1 h and then incubated over night at 4 °C with 1:100 specific primary antibody [IκBα: rabbit polyclonal (sc-847), NF-κB p65: rabbit polyclonal (sc-109), p53: mouse monoclonal (sc-126), MLH1: rabbit polyclonal (sc-581); Santa Cruz Biotechnology, USA.] in 1% BSA. After washing with 1X PBS, slides were incubated with FITC conjugated specific secondary antibody (Santa Cruz Biotechnology, USA.) for 1 h in the dark at room temperature. Cells were then counter stained with 1 μg/ml DAPI (4′,6-diamidino-2-phenylindole, fluorescent nuclear staining dye) and visualized under fluorescence microscope (Leica DM 4000 B, Germany). For negative control same procedure was followed without addition of primary antibody. Immunopositive cells were scored according to Perrone et al., 2006 based on their localization [nucleus (N); cytoplasm (C); both nucleus and cytoplasm (B)] and staining intensity [low, moderate and high]. Expression of specific protein was quantified according to localization and staining intensity of majority of cells of a subject. Sample percentages were made and plotted against their localization and staining intensity. The expression levels of subjects in each age group were compared with expression level of control subjects of respective age groups.

2.8. Statistical analysis

Student's t- test was performed to compare the ROS levels of different groups with respect to control subjects of respective age groups. Analysis of variance (ANOVA) (One way ANOVA) was performed to compare individually the TD% and OTM of different groups with respect to control group of respective age groups. Mean val-

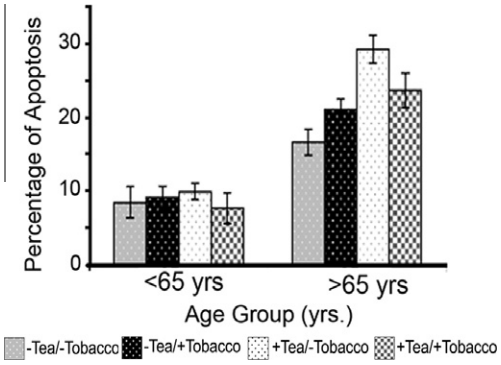

Fig. 3. Graphical representation of apoptosis percentage in subjects of<65 y and > 65 y age groups with/without tobacco and/ or tea habits.

ues of ROS generation, TD%, OTM and apoptosis percentages were graphically presented as mean ± standard deviation (SD). The p values < 0.05 and <0.01 were considered as statistically significant. Multivariate logistic regression analysis (enter method) had been done to analyze the effect of different factors like age, tobacco habit and tea habit on ROS generation and DNA damage with the help of statistical package Epi info (version 6, center for disease control and prevention). However, gender was not considered as a factor because there was an evident disproportion among male and female tobacco users in the subjects under consideration.

3. Results

3.1. Effect of black tea habit on ROS level in exfoliated buccal cells

Control subjects of older age group (>65 y) showed comparatively high mean ROS level (8.53 ± 0.88) than control subjects of < 65 y age group (4 ± 1) (Fig. 1). ROS level was significantly higher (9.3 ± 1.4) (p value < 0.05) in tobacco users of <65 y age group than respective control. Similar trend (11.2 ± 0.8) was also seen in tobacco users of >65 y. age group. Subjects with only tea habit showed comparable mean ROS level with respect to the control subjects of both age groups. However, significantly lower ROS level (4.9 ± 0.9) (p value < 0.05) was observed in tobacco users with black tea habit than only tobacco users in <65 y age group. Similar trend (9.3 ± 0.5) was also seen in older age group. In subdivided age groups (<20, 25–40 and 45–60 y) of <65 y similar pattern of ROS generation was observed in the respective groups (data not shown).

3.2. Effect of black tea habit on DNA damage in exfoliated buccal cells

Differential comet patterns were seen in the different subjects with/without tobacco and tea habit (Fig. 2A). High tail DNA percentage was seen in case of only tobacco users. Subjects having tea habit showed comet with larger head DNA than subjects with tobacco habit. Interestingly, subjects with both tea and tobacco habit showed comet pattern with larger head DNA and smaller tail DNA than subjects with only tobacco habit (Fig. 2A).

One way ANOVA showed that in both age groups tobacco habit significantly increased (p < 0.01) the tail DNA percentage (TD%) [<65 y (59.58 ± 4.18) and >65 y (61.86 ± 4.64)] than respective control group [(40.46 ± 6.34) and (46.09 ± 3.8) respectively] [(F_{3608} = 992.47; p < 0.01) (F_{3592} = 666.38; p < 0.01) F values respectively] (Fig. 2B). However, tea habit significantly (p < 0.01) decreased the TD% in tobacco user group [(39.82 ± 6.71) and (46.63 ± 4.28) respectively] than the subjects with only tobacco habit in both age group (Fig. 2B).

Similar trend was also found in case of Olive tail moment (OTM). In both age group tobacco habit significantly increased (p < 0.01) the OTM [<65 y (8.66 ± 0.76) and >65 y (8.74 ± 0.76)] than respective control group [(6.33 ± 0.84) and (7.21 ± 0.79) respectively] [(F_{3608} = 510.308; p < 0.01) (F_{3592} = 216.60; p < 0.01) F values respectively] (Fig. 2C). However, tea habit significantly (p < 0.01) lowered the OTM in tobacco user group [(6.62 ± 0.77) and (7.45 ± 0.8) respectively] than the subjects with only tobacco

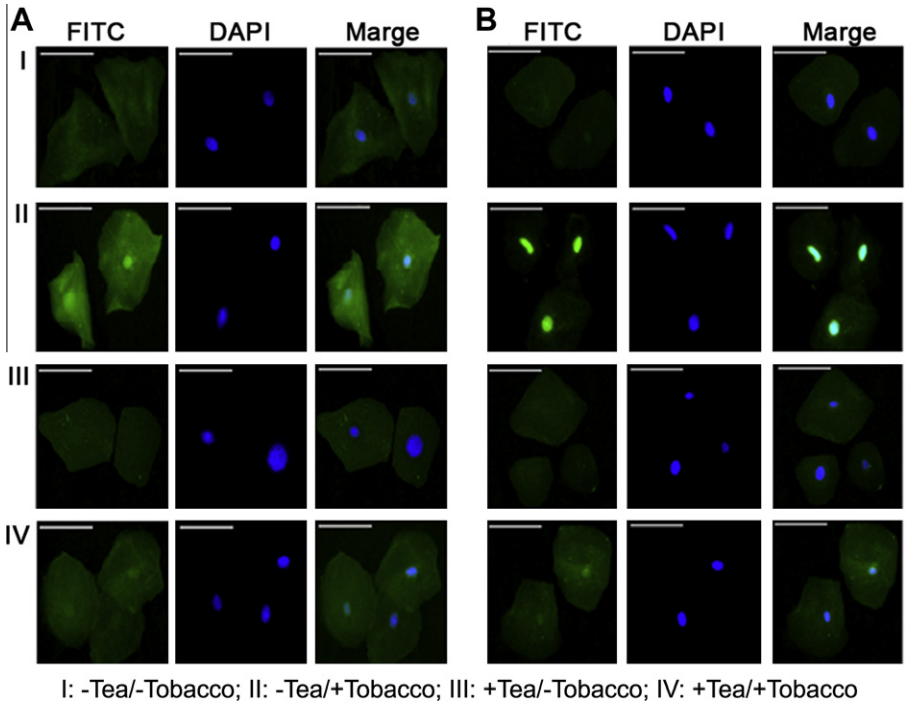

Fig. 4. Representative photographs of IκB (A) and NF-κB (B) expression by immunocytochemistry in different groups. (A) I, very weak cytoplasmic expression; II, strong nuclear and cytoplasmic expression; III, weak cytoplasmic expression; IV, low nuclear and cytoplasmic expression; (B) I, undetectable expression; II, strong nuclear expression; III, weak cytoplasmic expression; IV, low nuclear and cytoplasmic expression. (40× magnifications and scale bar 50 μm).

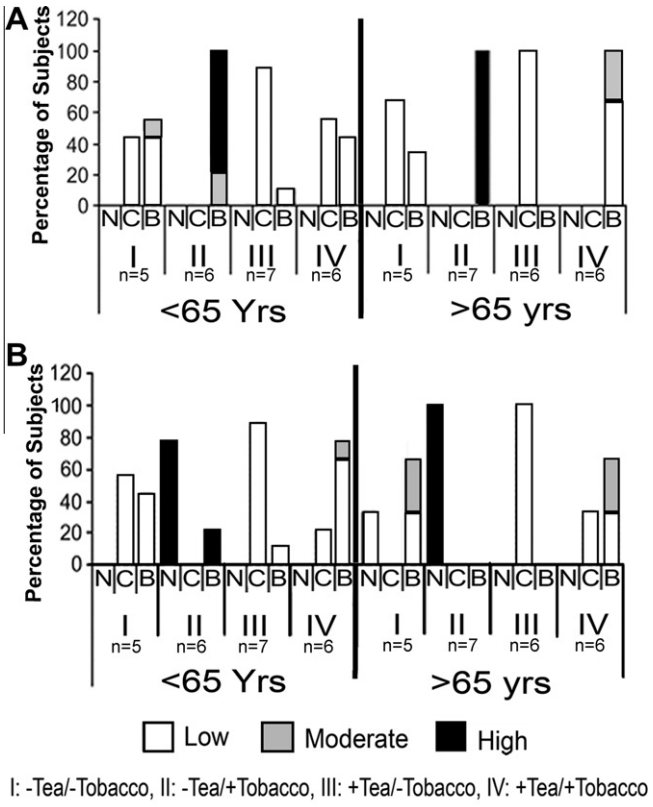

Fig. 5. Graphical representation of percentage of subjects showing expression of ROS related proteins (A) IκB; (B) NF-κB vs their localization [nucleus (N); cytoplasm (C); both nucleus and cytoplasm (B)] and staining intensity [low, moderate and high]. "*n*" represents the sample number in different sub-groups.

habit in both age group (Fig. 2C). Like ROS level, in <65 y subdivided age groups (<20, 25–40 and 45–60 y) similar pattern of TD% and OTM were observed [data not shown].

3.3. Effect of black tea habit on apoptosis in exfoliated buccal cells

In the <65 y age group percentage of apoptotic cells was low in the control subjects as well as in the subjects with/without tobacco and/ or tea habit (7–9%) (Supplementary Fig. 1 and Fig. 3). In case of >65 y age group, percentage of apoptotic cells was comparatively higher in the control subjects (17 ± 2.8%) and slight increase in apoptosis was observed in rest of the subgroups.

3.4. Effect of black tea habit on expression of IκB and NF- κB in exfoliated buccal cells

Differential expression patterns of IκB and NF-κB were seen in control subjects and the other groups (Fig. 4). In control subjects, low expression of IκB was seen either in cytoplasm (44–66%) or in both nucleus and cytoplasm (55–33%) in both the age groups (Fig. 5A). However, high nuclear and cytoplasmic expression (77–100%) of IκB was observed in only tobacco users irrespective of age groups. On the contrary, the subjects with only tea habit showed low cytoplasmic expression (88–100%) of IκB in both the age groups. Interestingly, tea habit lowered the expression of IκB in tobacco users irrespective of age groups. In <65 y age group either low cytoplasmic (55%) or both nuclear and cytoplasmic (44%) expression were observed, whereas in >65 y age group low to moderate expression in nucleus and cytoplasm was seen (Fig. 5A).

In case of NF-κB, control subjects of <65 y showed low expression either in cytoplasm (55%) or in nucleus and cytoplasm (44%). In control subjects of >65 y age group, low to moderate expression was seen in nucleus and cytoplasm (66%) along with low nuclear expression in some cells (33%) (Fig. 5B). In tobacco users intense nuclear expression of NF-κB (77–100%) was observed irrespective of age groups. On the other hand, low cytoplasmic expression of NF-κB was seen in the subjects with only tea habit irrespective of the age groups. However, tea habit could reduce the expression of NF-κB in both groups and its localization was observed in both nucleus and cytoplasm (Fig. 5B).

3.5. Effect of black tea habit on expression of p53 and MLH1 in exfoliated buccal cells

Differential expression patterns of p53 and MLH1 were observed in different groups (Fig. 6). In control subjects, low cytoplasmic expressions of p53 (86–66%) were seen in majority of subjects of both age groups. In addition, both nuclear and cytoplasmic expressions of p53 were seen in few subjects (33%) of older age group (Fig. 7A).High nuclear (82–100%) expression of p53 was observed in only tobacco users in both age groups. On the other hand, low cytoplasmic expression was seen in the subject with only tea habit irrespective of age group. Interestingly, tea habit could reduce the expression of p53 in tobacco users of both age groups and its expression was localized in nucleus and/ or cytoplasm (Fig. 7A).

In case of MLH1, control subjects of <65 y age group, showed low nuclear (22%) and low to moderate cytoplasmic expression (66%) (Fig. 7B). Whereas, in >65 y age group control subjects showed low to moderate nuclear (66%) expression and low cytoplasmic (33%) expression. However, intense nuclear expression (77–100%) of MLH1 was seen in only tobacco users of both age groups. On the contrary, low cytoplasmic expression of MLH1 was seen in only tea users irrespective of age group. Interestingly, tea habit could reduce the expression of MLH1 in tobacco users in both age groups and its expression was localized in nucleus and/or cytoplasm (Fig. 7B).

3.6. Association of different factors with ROS level and DNA damage in exfoliated buccal cells

The subjects having ROS above 5.0 [maximum range of ROS in subjects with no tea and tobacco habits without considering age group] were considered as cases and the rest were considered normal. Similarly, subjects having tail DNA percentage more than 43% [maximum range of TD% in subjects with no tea and tobacco habits without considering age group] were taken as cases and the rest were considered as normal. Assessment of risks of ROS generation and DNA damage were calculated separately for the age group <65 y compared to >65 y; having tobacco habit compared to not having tobacco habit and having tea habit compared to not having tea habit. Respective Odds ratios are given in Table 2. This data revealed that subjects with tobacco habit showed 2.86 times high risk of ROS generation [*OR* = 2.86 (1.79, 4.55)] and 15.36 times high risk of DNA damage [*OR* = 15.36 (8.38, 28.16)]. On the other hand, tea habit showed no risk of ROS generation [*OR* = 0.59 (0.37, 0.93)] and DNA damage [*OR* = 0.74 (0.46, 1.18)]. Similarly risk of ROS generation [*OR* = 0.2 (0.1, 0.37)] and DNA damage [*OR* = 0.46 (0.26, 0.81)] were not associated with age group.

These data were further confirmed by multivariate logistic regression analysis (enter method) for ROS generation and DNA damage separately which also reflected the subjects with tobacco habit to be 3.7 times at high risk for ROS generation [*OR* = 3.7

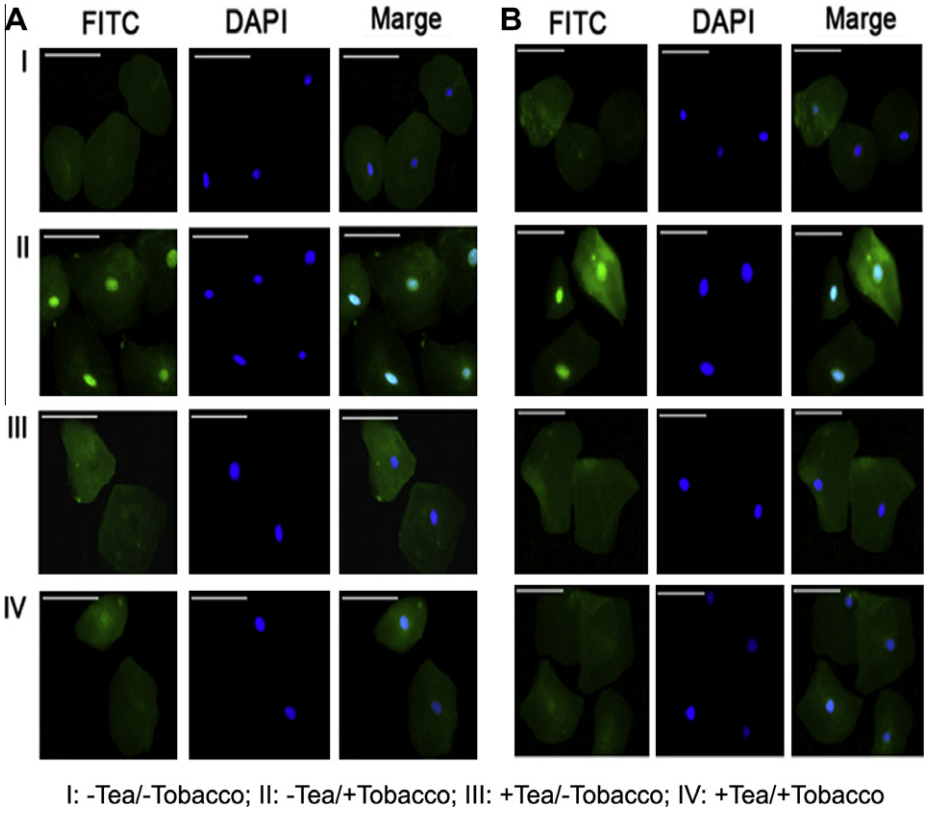

Fig. 6. Representative photographs of p53 (A) and MLH1 (B) expression by immunocytochemistry. (A) I, undetectable expression; II, strong nuclear expression; III, weak cytoplasmic expression; IV, weak cytoplasmic expression; (B) I, very weak cytoplasmic expression in control sample; II, strong nuclear and cytoplasmic expression; III, weak cytoplasmic expression; IV, weak cytoplasmic expression (40× magnifications and scale bar 50 μm).

(2.2, 6.3)] and 21.6 times at high risk for DNA damage [*OR* = 21.6 (10.9, 42.6)]. Tea habit showed no risk of ROS generation

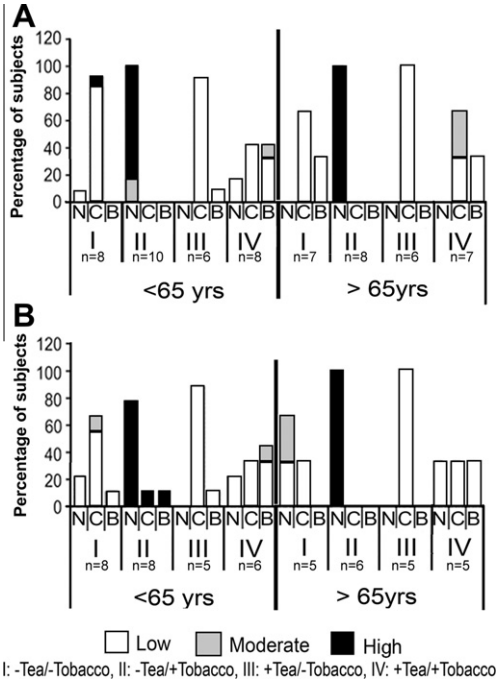

Fig. 7. Graphical representation of percentage of subjects showing expression of DNA damage associated proteins (A) p53 (B) MLH1, vs their localization [nucleus (N); cytoplasm (C); both nucleus and cytoplasm (B)] and staining intensity [low, moderate and high]. "*n*" represents the sample number in different sub-groups.

Table 2
Risk assessment of different factors against ROS generation and DNA damage.

Outcome	Exposure variable	Odds ratio [a]	95% Confidence interval (CI)		Odds ratio [b]	95% Confidence interval (CI)	
			Lower	Upper		Lower	Upper
ROS generation	Age	0.2	0.1	0.37	0.14	0.07	0.29
	Tobacco	2.86	1.79	4.55	3.74	2.22	6.28
	Tea	0.59	0.37	0.93	0.42	0.25	0.70
DNA damage	Age	0.46	0.26	0.81	0.25	0.11	0.54
	Tobacco	15.36	8.38	28.16	21.55	10.90	42.62
	Tea	0.74	0.46	1.18	0.42	0.23	0.78

[a] Odds ratio calculated by univariate analysis.
[b] Odds ratio estimated by logistic regression (enter method) analysis.

[*OR* = 0.41 (0.25, 0.7)] and DNA damage [*OR* = 0.42 (0.23, 0.78)]. Similarly, age group also not a risk factor for ROS generation [*OR* = 0.14 (0.07, 0.39)] and DNA damage [*OR* = 0.25 (0.11, 0.54)] (Table 2).

To further confirm the beneficial role of regular tea habit in case of tobacco users, the database were divided into two groups: subjects with tobacco habit; without tobacco habit and above mentioned statistical analyses were done. Surprisingly, no risk were observed for ROS generation [*OR* = 0.25 (0.12, 0.50)] and DNA damage [*OR* = 0.56 (0.28, 1.11)] in case of subjects with both tobacco and tea habit (data not shown in the table).

4. Discussion

This is a population based study to understand whether black tea habit could protect from the tobacco induced changes in the

buccal mucosal cells. In the control subjects of older age group (>65 y) comparatively high ROS level and DNA damage frequency were seen than in the younger group (<65 y). This might be due to inefficient xenobiotic detoxification due to cellular senescence resulting in accumulation of DNA damage (Di Domenico et al., 2010). Chronic tobacco habit generates constant ROS in the buccal cavity of tobacco users resulting in DNA damage in any age group (Haveric et al., 2010). We noted that regular black tea habit was significantly associated with lower ROS level and severity of DNA damage in tobacco users of all age groups. Furthermore, our data suggests that regular black tea habit could also lower the ROS level and DNA damage frequency to some extent in non-tobacco users also. Different *in vitro* and *in vivo* analysis revealed the antioxidative potential of black tea polyphenol and black tea extract (Yang et al., 2009; Frei and Higdon, 2003). Several epidemiological studies also revealed the effect of black tea consumption on plasma and urine antioxidant activity (Hakim et al., 2003). To the best of our knowledge no population based analysis has been done so far to understand the antioxidative potential of black tea in buccal cells of tobacco users.

Apoptosis was analyzed in buccal cells to differentiate between the tobacco associated DNA damage and apoptosis. Comparable level of apoptosis was seen in all subjects except older age group indicating tobacco associated DNA damage and apoptosis as two independent phenomenons (Liu et al., 2005). However, comparatively high frequency of apoptosis seen in the older age group might be due to inefficient DNA repair mechanism (Di Domenico et al., 2010). Green tea was reported to increase apoptosis in buccal cells of smokers (Chung et al., 2003).

Expression of IκB and NF-κB were studied in the buccal cells as a cytological marker of ROS generation. Lower expression of these proteins was observed in control subjects and the subjects with only tea habit in all age groups. However, high nuclear and cytoplasmic expression of IκB and high nuclear expression of NF-κB were evident in buccal cells of only tobacco users indicating the importance of these proteins as cytological markers of ROS generation (Van der Berg et al., 2001). It seems that constant ROS generation in the buccal cells due to chronic tobacco exposure might activate NF-κB, translocated to nucleus, and transcription of target genes along with IκB for feedback regulation (Sachdev et al., 1998). Newly synthesized IκB could enter into nucleus by its nuclear import sequence and inhibit NF-κB dependent transcription (Sachdev et al., 1998). Low expression of these proteins along with their changes in sub-cellular localization in the subjects with only tea or both tea and tobacco habits indicates its importance in modulating the tobacco associated alterations in buccal cells. It seems that black tea polyphenols could inhibit the ROS generation through activation of phase II detoxification enzymes (Frei and Higdon, 2003).

To further assess the role of black tea habit in prevention of oxidative DNA damage, the expression of two DNA repair associated proteins p53 and MLH1 were analyzed in buccal cells. Low expression of these proteins was observed in the control subjects of all age groups. However, high nuclear expression of p53 and MLH1 evident in only tobacco users suggest these as potential biomarkers of high DNA damage. It seems that in response to the oxidative stress and DNA damage due to chronic tobacco exposure there is stabilization of p53 protein and translocation to nucleus for transcription of different target genes for efficient DNA repair (Liang and Clarke, 2001). Similarly, high nuclear expression of MLH1 in the tobacco users is also required for DNA repair process; though the importance of its cytoplasmic expression is not fully clear (Ghosh et al., 2010). Reduced expression of p53 and MLH1 seen in the tea users with or without tobacco habit could be due to low induction of these proteins for low rate of DNA damage.

Finally the present study suggests that regular and 4–10 cups of drinking of black tea may be beneficial for reducing the risk of ROS generation and DNA damage in habitual tobacco users.

5. Conclusion

The Present study indicates a chemopreventive role of black tea against oral cancer by reducing risk due to tobacco induced ROS generation and DNA damage of buccal cells. Moreover, expression of ROS associated proteins IκB, NF-κB and DNA repair associated proteins p53 and MLH1 in the buccal cells may be used as cytological markers for detection of risk and risk reduction in normal population. This study also supports the contention that black tea may elicit health benefit by virtue of its anti-genotoxic effect.

Conflict of Interest

The authors declared that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fct.2012.06.005.

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