

Elucidation of baseline single stranded DNA breaks in smokers— Tiruchirappalli, India

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ABSTRACT

Smoking is a major contributing factor enhanced many health problems particularly lung cancer which has a survival percentage of 16.4% which increases the risk of cancer and other diseases in humans and animals. DNA damage is the cause of cancer as it damages the cellular apoptosis system severely that the injured cell cannot kill itself and become cancerous. Tobacco smoke also contains various carcinogens other than polynuclear aromatic hydrocarbons, such as traces of radioactive elements. The single – cell agarose electrophoresis (SAGE), popularly known as the Comet assay is a simple, versatile and visually pleasing technique that is widely being used in detecting quantifying DNA damage and repair. Present study were observed ageing leads to increase levels of SSBs breaks.

Key words: Smokers, Cancer, DNA damage, Population

INTRODUCTION

Cigarette contains nicotine, a stimulant which not only temporarily improves alertness, memory and mood but also forms a strong physical and psychological chemical dependence (addiction). Nicotine like any other stimulant can also increase anxiety, restlessness and disturb metabolism. Medical research has determined that tobacco smoking is a major contributing factor towards many health problems particularly lung cancer which has a survival percentage of 16.4% which increases the risk of cancer and other diseases in humans and animals [1,2]. Non-smokers are thought to live approximately 10 years more than the smokers. The damage a continuing smoker does to his lungs can take up to 20 years to physically manifest as lung cancer [3]. The carcinogenicity of tobacco smoke is not explained by nicotine which is not carcinogenic or mutagenic. Rather partially burnt material contains polycyclic aromatic hydrocarbons particularly benzopyrene whose oxidation produces an epoxide, which binds to DNA covalently and permanently distorts it. DNA damage is the cause of cancer as it damages the cellular apoptosis system severely that the injured cell cannot kill itself and become cancerous. In this respect the mechanism of damage closely resembles that of mustard gas, aflatoxin and other DNA alkylating agents [4].

Tobacco smoke also contains various carcinogens other than polynuclear aromatic hydrocarbons, such as traces of radioactive elements. For example, smoke from tobacco grown with phosphate fertilizers contains polonium 210 [5]. It is estimated that the estimated risk of cancers caused by P210 is 4 per 10,000 smokers [6].

The carcinogenicity is aggravated by the delivery of the carcinogens, namely direct inhalation. Radioactive and carcinogenic particles would not find their way by itself to the lungs but a smoker deliberately inhales them repeatedly over a long period of time. Thus there is a good correlation between the incidence of lung cancer and smoking [7].

Our study is aimed at studying the single stranded DNA (SSB) breaks by comet assay, thereby giving a baseline frequency of SSBs in smokers.

MATERIALS AND METHODS

The population for control was selected based on clean habits such as non-smoking and non-tobacco use. The sample size was 5 per group and total of 30 for the six groups. The smokers were asked for the number of cigarettes smoked per day and only those who consumed more than 10 cigarettes per day were included in the study. The duration of smoking was from 3 years in the <20 years age group who had taken up the habit in their late teenage and continued thereon. No smoker who had broken the habit even for a brief period of time was included in the study. An informed consent was obtained from all the subjects involved in the study.

Separation of lymphocytes

Lymphocytes were separated from peripheral blood by density gradient method using Histopaque -1077. Three ml Histopaque was taken in a conical test tube and 3 ml of blood sample was carefully layered on the above solution. This was centrifuged at 2200 rpm for 30 min. Erythrocytes and the granulocytes sedimented at the bottom of the centrifuge tube, while lymphocytes formed a buffy coat over the Histopaque layer. The upper layer contained plasma, the buffy coat was carefully aspirated with a Pasteur pipette and carefully transferred to a conical tube and the plasma was used as serum supplement in all the experiments. The lymphocytes obtained were washed with 6 ml of PBS and centrifuged at 1200 rpm for 10 min. The cell pellet was again washed with PBS for another 4 times as above. For the comet experiments, 1 ml of the blood sample was over laid on 1 ml of Histopaque solution and lymphocytes were separated as above.

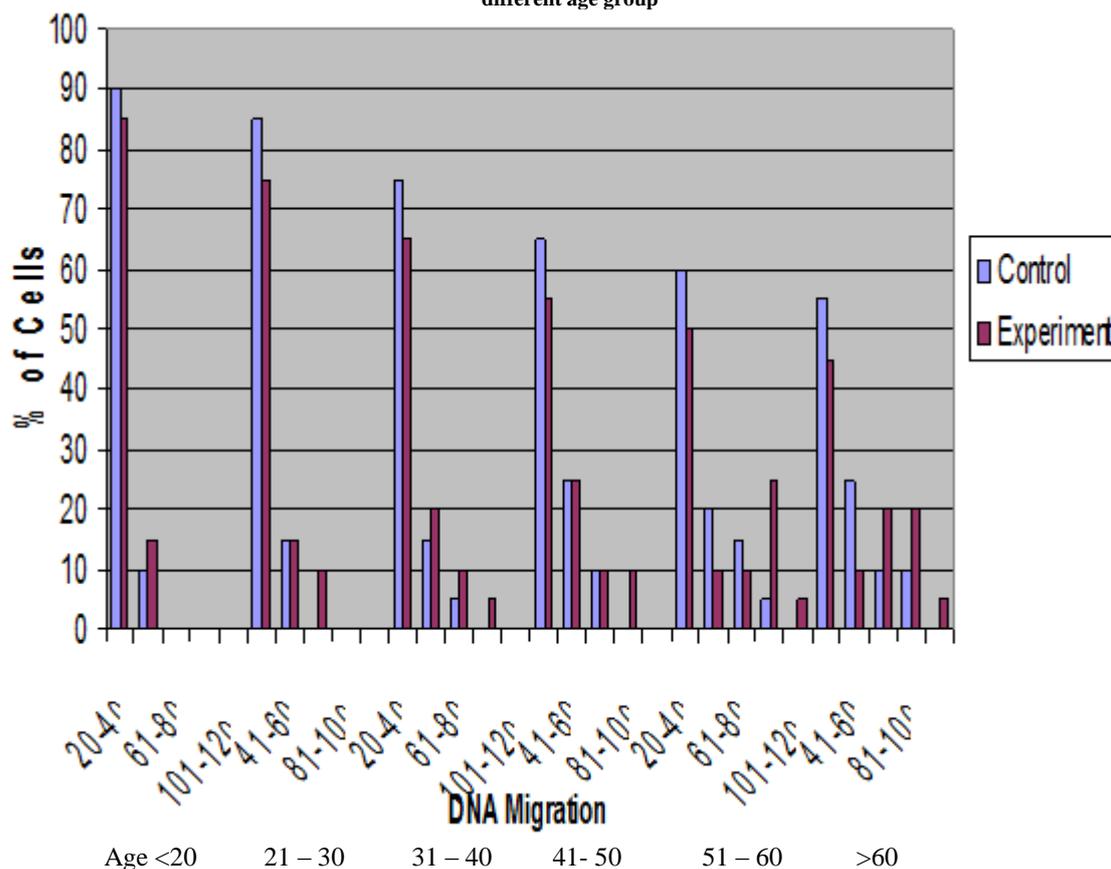
Comet assay or single-cell agarose gel electrophoresis (SAGE) :

The single – cell agarose electrophoresis (SAGE), popularly known as the Comet assay is a simple, versatile and visually pleasing technique that is widely being used in detecting quantifying DNA damage and repair [14]. Comet's tail observed under the microscope were measured using an ocular micrometer (OM) and a stage micrometer (SM).

RESULTS

The various age groups of controls and smokers with the relevance to comet formation were tabulated. The tests yielded large amount of data in the form of comet tail lengths with regard to age groups. The overall view present by the experiment was that as age increases the damage, namely single stranded breaks increases and in other words they are directly proportional and reached a peak value of damage above 60 years of age for both the groups. In controls the SSBs as envisaged by comet assay progressed from 1% at the age of <20 years age group to 8% above the age of 60 years, which correlates to about eight fold increase in such damages. However, the smokers were seen to have about 3% SSBs even at <20 age group and progressed to about 18% damaged cells above 60 years age group. This means that to begin with the damaged cells were three times more than those in the control population and ended up with six fold increase compared to themselves and eighteen fold increase to that of the controls respectively (Fig. 1). The exponential trend line (regression analysis) plotted based on the two groups is shown in Fig 2. Based on the exponential regression analysis the calculation of p-values were done and the result showed that the significance levels were $p > 0.05$ for the first two age groups <20 and 21-30, with the middle age groups of 31-40 and 41-50 showing high significance difference of $p > 0.001$, followed by the last two age groups of 51-60 and >60 groups to be highly significant with p values > 0.0001 . The Fig,2 shows that the controls exhibit inverted linear quadratic graph, while it is inverse sigmoidal in case of the smokers.

Fig. 1 Histogram showing the distribution of the length DNA migration in lymphocytes obtained from control and smokers population of different age group



DNA MIGRATION [Comet tail length in μm]	Age <20		21 – 30		31 – 40		41- 50		51 – 60		>60	
	C	E	C	E	C	E	C	E	C	E	C	E
20-40	85%	90%	75%	85%	65%	75%	55%	65%	50%	60%	45%	55%
41-60	15%	10%	15%	15%	20%	15%	25%	25%	10%	20%	10%	25%
61-80	-	-	10%	-	10%	5%	10%	10%	10%	15%	20%	10%
81-100	-	-	-	-	5%	-	10%	-	25%	5%	20%	10%
101-120	-	-	-	-	-	-	-	-	5%	-	5%	-

DISCUSSION

The DNA of the smokers have a general tendency to “break”, i.e. the double strands, the cross links and other such forces that hold the DNA together tend to disrupt automatically without any external stimuli. This phenomenon is called the “Fragile Syndrome” and has been studied extensively using several techniques as well as by many researchers. The reason is not yet clearly understood, even though the fact that whether this is the cause for lung cancer predisposition or because of lung cancer such fragility arises is also not clearly understood. However, the fact remains that these people (lung cancer patients) tend to develop several syndromes associated with genetics and biochemical disorders. Thus, the basic aim of our study was to characterize the baseline damages in smokers.

While the smokers have inhaled the pleasures of tobacco they pay by means of SSBs, we have created a baseline data for the exposure to smoke vs age.

The control values obtained by the comet assay for those samples non-smokers and smokers the cells were mostly (~99%) are intact with no obvious comet formation. This indicates primarily that the smoker lymphocytes do not show much SSBs even though they are said to be fragile at the beginning of such a vice. Secondly, the presence of environmental mutagens did not enhance the level of baseline damage seen in the cells, even though the control or non-smokers are constantly exposed to various levels of other pollutants. This is vital because if environmental factors itself induces such damages then it may not be viable to study this in detail.

The induction of SSBs, when the cells are obtained from older people clearly indicated that these cells were predisposed to such damage and then the rise in damage is directly proportional to the incurred dose showed a linear

dose response. This reached a peak when the age reached >60 years. This finding validates the concept of ageing. That is older persons form more comets length. This point is designated as Point Of Decline (POD) in the controls and is seen to be the beginning of a steep fall in the number of good cells, having more or less intact nucleus. However, in case of the smokers such a decline begins at the age group of 31-40 itself, indicating that even though there is no Fragile Syndrome in the smokers, smoking induces certain fundamental changes in the cellular metabolism that give rise to SSBs at an earlier stage of life itself, that may or may not predispose the individual to further moribund or adverse conditions [12,13].

Similarly, reported that the damage to DNA in lymphocytes can be related to the exposure to petroleum fuels to increase in the olive tail moment [10,11] and Clearly demonstrated an increase in sperm double-stranded DNA breaks with age and also suggest for the first time an age-related decrease in human sperm apoptosis. Reported the DNA damage among the workers occupationally exposed to lead - peripheral blood lymphocyte shows increased levels of DNA damage of cigarette smoking workers has a synergistic effect on inducing DNA damage [9]. Reported that the difference in DNA damage between smokers and non-smokers was statistically significant [8].

The graph was constructed with values of both non-smokers as well as smokers as a percentage of cells versus comet tail lengths which gives a dose-response curve Fig 2. The resultant graph gives rise to inverse linear-quadratic and sigmoidal graphs for controls and smokers, respectively.

The crux of the work is that it elucidates the levels of SSBs in both non-smokers and smokers into a Dose-Response curve in terms of age. This helps in two major and one minor aspects of the study in case of diagnosis and prognosis of lung cancer and other different kinds of respiratory diseases seen in smokers. The way by which this is achieved is as follows:

- a) the non-smokers and smokers trend line gives the difference between two groups and shows that the level of difference is significant at $p > 0.05$ level in t-test. This means that if a smoker is tested for his comets in lymphocytes the findings indicate the levels of SSBs as per the curve then his smoking habit is ascertained.
- b) The level if exceeds that of the normal limits as seen from this study the subject may show predisposition towards lung cancer.
- c) The same test if performed after treatment of lung cancer and if the values are nearer to normal ones the patient is said to be recovering well and if it tends to deteriorate the prognosis is bad and remission could be expected.

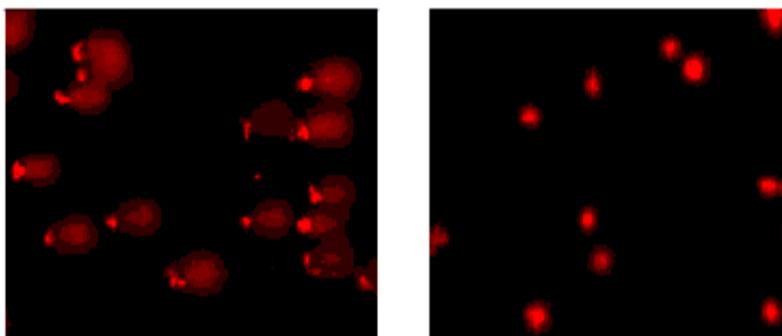
The following salient features emerge from the present study:

- i) Smokers peripheral blood lymphocytes are not entirely fragile
- ii) Ageing leads to increase levels of SSBs.
- iii) Samples of people >60 years show the maximum damage
- iv) Relative damage of ageing and smoking is seen to follow a linear trend.

CONCLUSION

The present study summarizes that the basic tenets of biology as well as the oncology are amenable to intelligent assays such a comet assay. It would also be appropriate to show that such study would lead to the development and validation of good techniques for enabling the betterment of the lives of the cancer patients.

COMETS STAINED WITH ETHIDIUM BROMIDE



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