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RESEARCH ARTICLE

ASSAY OF EXFOLIATED AND ASSOCIATED NUCLEAR ANOMALIES AS BIOMARKER IN TOBACCO EXPOSED GROUP

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ABSTRACT

Aim- Oral cancer is becoming the most common disease worldwide, rapidly increasing in tobacco exposed persons. We have studied the morphological as well as nuclear abnormalities in the buccal exfoliated cells in tobacco exposed persons in different forms.

Method- The technique involves extermination of epithelial smear from oral cavity of four experimental groups like smoker, chewer of smokeless tobacco, smoker with chewing habits with respect to control. Subsequent nuclei staining for determination of prevalence of cells containing micronucleus and meta-nucleus and other nuclear anomalies can be classified as genome damage parameter and cell death parameters and both the parameters were observed in the mentioned experimental groups. It is a non- invasive direct techniques and repeated sampling is easier; therefore suitable for epidemiological studies.

Result- The result shows significant amount of nuclear anomalies in tobacco exposed person in varying form as smoked and smokeless tobacco consumers in comparison to non exposed group. The result shows that, genome damage and cell death parameter has been found in elevated frequencies in tobacco chewer with smoking habits in comparison to the control one and the frequency of nuclear anomalies are 72.41±8.5% The smokeless tobacco chewers showed nuclear anomaly frequency in the range of 46.05±6.9% and only smoker groups shows the frequency of nuclear anomaly is 18.93±3.4.

Conclusion- From the data as obtain from this study, it can be concluded that nuclear abnormalities occurs predominantly due to the exposure of tobacco, from which severity of DNA damage can be easily predicted successfully. So the risk of cancer onset may be avoided employing these simple techniques.

INTRODUCTION

Cancer is still a main cause for mortality worldwide despite an intense and in depth research during the past decade. It is predicted that by 2020, the world population will increase to 7.5 billion; of which, approximately 15 million new cancer cases may be diagnosed, and about 12 million cancer patients may expire (Brayand, 2006). It is suggested that as a cause for cancer both internal factors (Genetic, Endocrinological and immunological) and environmental/acquired factors (such as tobacco, diet, radiation, and infectious organisms) are equally important. The link between diet and cancer remains a common cause in occurrence of specific type of cancers in different countries and also in the incidence of cancer in specific migrating populations (Anand, 2008).

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Most cancers are not of hereditary origin and lifestyle factors, such as dietary habits, smoking, alcohol consumption, and infections sometimes too show a profound influence on these cancer developments (Czene *et al*., 2002). According to world health organization, tobacco alone may cause more than 100 million deaths over the course of the 20th century worldwide (WHO, 2008), thus become a choice of serious study and research in recent periods. Tobacco plant, *Nicociana tabacum,* belongs to the family *solanaceae,* is native of America. Tobacco is cultivated in many regions around the world and can be legally purchased in all countries. The dried leaf of *Nicotiana tabaccum* is used for smoking, chewing and snuff. Tobacco use can be classified under six principal heading such as Cigarettes, Bidis, Cigars, Pipe tobacco, Snuff and Chewing. There are different forms which are usually taken orally comes under the common group of smokeless tobacco (Table-1). Smoking is also classified in four major groups and the detail of these four types is depicted in Table-2.

According to World Health Organization, globally, about one out of three adults smoke and a prediction shows that there will be an expected 1.6 billion smokers in the world by 2025 (Tobacco, 2006). If current trends persist, it has also been estimated that tobacco use will result in the death of more than one billion people worldwide in this century, and while a large majority of them will be due to smoking cigarettes, use of tobacco in other forms also contributes to the projected outcome (Tobacco, 2006). Over the time due to so called different positive physiological effects like diminishing hunger, heightened alertness, concentration increase and easily concealing use, smokeless tobacco(ST) gained popularity and due to the easy consumption procedures and social acceptance along with indoor smoking ban made these kind of smokeless tobacco use more agreeable (Mhaisekar, 2015). Over recent years, tobacco use and the ST market place has changed dramatically with the introduction of varieties of ST products. The National Sample Survey data indicate that, in India there are about 184 million tobacco consumers of which some 96 million people use smokeless tobacco, while 20% consume cigarettes, nearly 40% smoke bidis and remaining 40% chew tobacco (Kumar, 2000; Bahreinifar *et al*., 2013; Changrani *et al*., 2005; http://www.cdc.gov/ tobacco/data_statistics/ fact_sheets/smokeless/betel_quid.2014). Given the growing prevalence and global use of varieties of tobacco products, the current study has been taken to investigate the toxicological implications of tobacco in varying forms in exposed group with a comparison to the non exposed group using micronucleus and broken egg as a biomarker of genome damage and pyknosis, karyohexis, karyolysis as a biomarker of cell death. Assessment of micronucleus and other nuclear anomaly in exfoliated buccal cells are frequently used as a non invasive method to detect precancerous as well as cancerous lesions for monitoring the effects of a number of chemo preventive agents by a number of researchers (Stich *et al*., 1984; Stich, 1988 and Holland, 2008).

MATERIALS AND METHODS

Selection of Subjects

Study was done on the people living in Badu, South 24 parganas, and its adjoining areas in West Bengal, India. The sample were collected from 50 smokers (Group-1), 50 smokeless tobacco chewer (Group-2), 50 tobacco chewer with smoking habits (Group-3) and 50 non tobacco consumers (Group-4). The first three groups were taken as case group and the last one or the fourth group was considered as control group. All the volunteers in both cases as well as control group were men. All the three case groups are categorized into two categories. In the first category or Category-1 consist of individuals whom Mean duration of Tobacco use (Year) =5. The second category or Category -2 consists of individuals whom Mean duration of Tobacco use (Year) =10. The age of the individuals in all four groups were ranging from 22-47years (Mean=29.25±2, N=200). At the time of sample collection sex and duration of exposure of the individuals in details was not considered and grouped as less than five year and more than five year and so on. The total study designs are clearly depicted in Table-3.

Sample Collection

Prior to collection of the samples the mouth of the volunteers were washed with water twice and then the buccal swab were collected carefully with the help of pre-moistened wooden spatula and making a smooth smear on the grease free glass slide. Informed consent was taken from all volunteers mentioning the purpose and impact of the study.

Slide preparation and staining

The collected sample was spread over the slides with a needle and the slides were then heat-fixed by placing it over a hot plate and dipped into alcohol. The slides were again dried by placing it over the hot plate and then stained with 4% giemsa stain (Sigma- Aldrich-SLBF4227) diluted with phosphate buffer in the ratio of 1:1and kept it for 5 minutes. The stained slides were washed with distilled water, air dried, mounted with DPX.

Observation

Prepare slides were observed under light microscope (Moticam) with 10X40 magnification. Five fields were observed randomly for counting the different anomalies. 200 cells from each individuals has been calculated under microscope and different anomalies were counted by a single observer for each exposed group at least five samples were taken. Anomalies were divided into two major groups like genome damage parameters and cell death parameters. Short description of each parameter was described in Table- 4.

Statistical Analysis

At the end of study, all the data were complied and tabulated using Microsoft Excel spreadsheet and Microsoft Office both of which are part of Microsoft Office enterprise 2007. Data were presented in the form of tables, bar graph, pie chart etc as needed. Analysis was done using appropriate and suitable statistical methods manually and using software including Microsoft Office Excel 2007. Observed nuclear abnormality, both the genome damage and cell death parameters, doing contingency chi square test for four groups to determine that all the attributes was independent or associated taking P- value less than 0.05 were considered statistically significant.

RESULTS

The nuclear as well as cellular anomalies as mentioned above were observed and scored. Different nuclear anomalies observed during study periods are clearly shown in Figure-1. Mean percentage of the nuclear anomalies in each study group is shown in Table-5. In the present study, the result analysis revealed that Tobacco chewer with smoking habits or Study group-3 had significantly the highest frequency of all nuclear anomalies compared to other three study groups. Smoker and smokeless tobacco chewer also had significant amount of nuclear anomalies in compare to normal control, but smokeless tobacco chewer shows more nuclear anomalies than smoker group. The graphical representation of genome damage and cell death parameters in four study groups are shown in Figure-2. The contingency chi square test show $8.538 > X^2_{0.05,3}=7.82$; therefore, null hypothesis is rejected and. alternative hypothesis is accepted. Therefore all four attributes are associated.

DISCUSSION

According to study, Indiaranks fourth in the total tobacco consumption percentage in the world comprising of all types

Table 1.Different forms of Smokeless Tobacco

SL No	Name	Forms	How to Use
1	Kheini	Dried and blended tobacco leaves	Leaves are mixed with slake of lime (Calcium Hydroxide) and placed between gum and cheek
2	Gutkha	Grinded tobacco leave with mixture of areca nut, slaked lime, catechu (extract from the wood of the Accacia plant) along with a number of spices	Taken orally and chewed
3	Zarda	Flake of dried Tobacco leave	Occasionally taken alone or chewed when mixed with betel leaf, lime and areca nut.
4	Snuff*	Finely ground Tobacco	Taken nasally

Table 2.Different forms of Smoked Tobacco

SL No	Name	Forms	How to Use
1	Cigarettes	Flake of Tobacco leave rolled by a fine paper	Smoked
2	Bidi	Flake of Tobacco leave rolled by a dried leave	Smoked
3	Ciger*	Flake of Tobacco leave rolled by a dried leave	Smoked
4	Hookka*	Powdered form of Tobacco	Smoked

*Though it is very common in Indian subcontinent but very much uncommon especially in our study area thus it could not be included in our study

Table 3. Design of the present study

Group	Type	Number of Individuals	
		Category-1	Category-2
Group-1	Smokers	25	25
Group-2	Smokeless tobacco chewer	25	25
Group-3	Tobacco chewer with smoking habits	25	25
Group-4	Non tobacco consumers	25	25

Table 4. Types and characteristics of different parameters used in the present study

SL No	Parameters	Types of Anomalies	Characteristics
1	Genome Damage parameters	Micronucleus(MN)	Smaller in size in the present nucleus with almost same staining intensity
		Broken Egg(BE)	Nuclear buds contain nuclei with an apparent sharp constriction at one end of the nucleus suggestive of elimination of nuclear material by budding
		Bi-nucleus (BN)	Presence of two nuclei within a cell with same staining intensity and size
2	Cell Death parameters	Karyohexis(KH)	Nuclear disintegration involving loss of integrity of the nucleus
		Karyolysis(KL)	Nuclear dissolution or ghost-like apperance of the nucleus
		Pyknosis(PK)	Shrunken nuclei with a high density of nuclear material that is uniformly but intensely stained

Table 5. Frequency of nuclear anomalies in buccal exfoliated cells of four study groups

Group	Parameters	Anomalies	Category-1	Category-2	Total % of anomalies Parameter wise	Total % of anomalies Group Wise
Smoker	Genome Damage	Micronucleus (MN)	2.03±2.9	2.43±2.0		
		Broken Egg (BE)	0.00±0.0	0.12±1.8	8.30±2.7	
		Bi-nucleus (BN)	1.54±2.8	2.18±3.0		18.93±3.4
	Cell Death	Karyohexis (KH)	0.01±0.2	0.02±0.6		
		Karyolysis (KL)	0.01±0.8	0.10±1.2	10.63±3.6	
		Pyknosis (PK)	4.37±6.9	6.12±4.3		
Smoke less tobacco chewer	Genome Damage	Micronucleus (MN)	2.94±1.5	3.80±2.8		
		Broken Egg (BE)	0.00±0.0	0.00±0.0	11.64±4.2	46.05±6.9
		Bi-nucleus (BN)	2.24±2.5	2.66±1.1		
	Cell Death	Karyohexis (KH)	3.48±4.9	6.49±7.8		
		Karyolysis (KL)	2.59±1.3	4.90±3.7	34.41±8.1	
		Pyknosis (PK)	8.13±2.3	8.82±6.1		
Tobacco chewer with smoking habits	Genome Damage	Micronucleus (MN)	6.69±6.8	9.20±9.6		
		Broken Egg (BE)	0.00±0.0	0.00±0.0	34.78±9.1	
		Bi-nucleus (BN)	8.93±1.3	9.96±5.2		72.41±8.5
	Cell Death	Karyohexis (KH)	3.76±2.4	4.01±7.8		
		Karyolysis (KL)	4.80±7.1	4.91±3.4	37.63±6.3	
		Pyknosis (PK)	8.78±4.5	11.35±5.7		
Control	Genome Damage	Micronucleus (MN)	0.92±0.8	0.96±4.3		
		Broken Egg (BE)	0.00±0.0	0.00±0.0	2.84±5.3	
		Bi-nucleus (BN)	0.44±0.1	0.50±1.8		6.22±1.2
	Cell Death	Karyohexis (KH)	0.33±0.1	0.04±0.2		
		Karyolysis (KL)	0.33±1.2	0.80±0.5	3.58±1.1	
		Pyknosis (PK)	1.01±0.2	0.87±0.3		

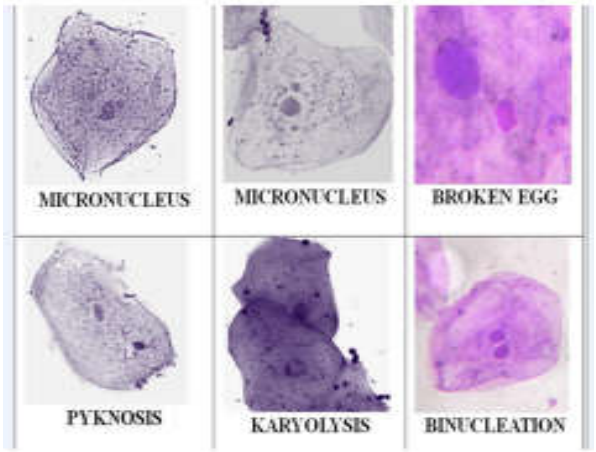

Figure 1. Showing Different nuclear anomaly in different tobacco exposed group

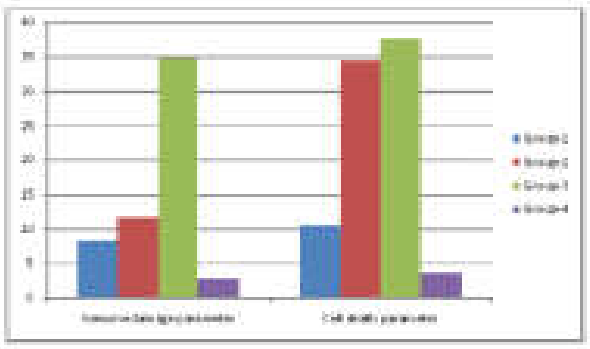

Figure 2. Compare the two parameters of nuclear anomalies in exfoliated buccal cells in four study groups

of ST and smoked tobacco forms. Chewing tobacco has been a tradition in India for centuries often as a mixture of nuts, seeds, herbs, and spices, which may correlates with a study that millions of Indian mostly young people are found to suffer from oral submucosa fibrosis- a disease which is precursor of oral cancer (Reddy, 2004). It has been shown in studies that approximately 90% of human cancers originate from epithelial cells (Rosin, 1992). Oral epithelial cells oftenassayed as a preferred target site for some early genotoxic events commonly induced by carcinogenic agents via these routes as buccal cells are thought to be the first barrier in the route of inhalation or ingestion and are capable to metabolize proximate carcinogens to reactive products (Autrup, 1985; Liu, 1993; Vondracek, 2001; Spivack, 2004).

Usually oral epithelium maintains its normal organization by continuous cell renewal process whereby new cells produced by mitosis in the basal layer replace the shed cells and later migrate to the surface. The basal layer along with the stem cells during nuclear division may express some genomic damages as chromosome breakage or Loss as genetic damage in form of Mocronucleus (MN). The daughter cells with or without MN, differentiate into the prickle cell layer along with the keratinized superficial layer, and finally exfoliateinto the buccal cavity. Few exfoliated cells may also degenerate into condensed chromatin, fragmented nuclei (karyorrhecticcells), pycnotic nuclei, form karyolitic or ‘‘ghost’’cells by losing their nuclear material completely (20) or may remain in a binucleated stage or form nuclear buds (alsoknown as ‘‘broken eggs’’)in buccal cells, a biomarker of geneamplification.

These biomarkers of genome damage (e.g.MNi, nuclear buds) and cell death (e.g. apoptosis, karyolysis) found in oral exfoliated cell systemsprovide a comprehensive assessment of genome damagein the area of cytotoxicity and often show cytostatic effects (Tolbert, 1992; Speit *et al*., 2006). As a moderate number of cells with degrading nuclei may also sometimes occur during normal cytotoxic processes and may not always be associated with clastogenic or aneugeneic DNAdamage (Tolbert, 1992; Titenko-Holl *et al*., 1996; Nersesyan *et al*., 2006). It is important to assess few parameters before considering a cell to be studied for cytotoxicity purpose. Those are- presence of intact cytoplasm and relatively mono layered cell position on the slide, little or no overlap with adjacent cells, little or no debris with nucleus normal and intact, nuclear perimeter, nucleus, a smooth distinct for proper evaluation of cancer risk (Tolbert *et al*., 1991). Since 1937 micronucleus has been used as an indicator of genotoxicity for genetic screening of a cytogenetic damage as (Jyoti *et al*., 2012; Bansal *et al*., 2012) studies on MN showed that MN frequencies increase as a product of early events in human carcinogenic processes, and could be used as an identification of preclinical steps of the cancer which could be used as an early awareness initiative (National cancer control programmes- policies and managerial guidelines, 2002).

Betel quid chewers from 1985 till date have been found a significant increase in micro nucleated frequency as compared with healthy individuals in a case control study(Nair *et al*., 1991) proves the efficacy of this parameter as a successful biomarker. In the present study, both the genome damage and cell death parameter has been found in elevated frequencies in tobacco chewer with smoking habits in comparison to the control one and the frequency of nuclear anomalies are 72.41±8.5% The smokeless tobacco chewers showed nuclear anomaly frequency in the range of 46.05±6.9% and only smoker groups shows the frequency of nuclear anomaly is 18.93±3.4. The genome damage and cell death parameters individually elevated in the subjects of group -3 and the percentages are 34.78±9.1% and 37.63±6.3% respectively. These findings clearly shows that, the oral epithelial cells are directly contact with genotoxic agent from smokeless tobacco category during chewing of STs and in case of a smoker, the toxic smoke come in contact with the oral epithelia during brief smoking period create minimum toxicity or nuclear anomaly in comparison to the smokeless tobacco form. But, when smoking adds with other smokeless tobacco products like khaini, gutkha or zarda then it causes highest percentage of nuclear anomalies as well as genetic instability as an accumulated or additional toxicity.

Carcinogenic pyrolytic compounds produced by tobacco smoking bind to DNA and create genetic alteration. A dose response relationship between the used tobacco product and oral cancer progression has already been established by the researcher. The whole oral cavities are susceptible to the carcinogenic effect of tobacco in both smoked and smoke less form. Detailed study of nuclear anomalies in buccal exfoliated cells can act as predictor of oral cancer and their association with tobacco. A study conducted by Beena Patel (Patel Beena, 2009) also stated that there was a significant p value (p=0.001) for the micronucleus frequency between the controls and tobacco chewers. According to our present study, the highest percentage of damage has been shown by the group which smokes and also takes *khaini* together. Literature study shows

that all the previous screening in oral mucosa was done either smokeless tobacco consuming group or smoker group but not in combined pattern. So, as per our knowledge these kind of detailed studies on the specific group of smokeless tobacco users are not available at this juncture of time. Detailed analysis and observation revealed that, genetic instability plays a key role in the formation of nuclear anomaly in oral exfoliated cells of tobacco exposed persons. According to some researchers all the mentioned nuclear anomalies are the sequential stage in the process of apoptosis where the broken eggs represent the first stage in apoptotic pathway and karyolysis or formation of ghost cells suppose to be the last stage. Thus the percentage of particular nuclear anomaly detection may also indicate the progressing stages of oral mucosa cancer. Thus the present study also shows that, the nuclear anomaly screening from oral mucosa cells will ensure the key role in the evaluation of mutagenicity and strategy making for primary prevention in future. Due to the accuracy and non invasive regular and recurrent simple tissue collection method of oral exfoliated cell screening, may act as an attractive tools and the technique gives us a ray of hope to the physician, clinician and medical personal.

Conclusion

The present work, a pilot study using genome damage and cell death parameters and other associated nuclear anomaly act as a biomarker before a cancer finally onset becomes important when large samples are analyzed. As the present technique is a noninvasive, cost-effective quick and easy population screening process, this is of immense importance in the present scenario to assess the early risks of developing oral cancer. The present study shows that, different forms of tobacco cause significant amount of genome damage as well as apoptosis of the oral exfoliated cells. Smokeless tobacco causes serious health hazards and elevated both the parameters of nuclear anomalies in several fold either individually or in association with smoked form in comparison to controls.

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