A Preliminary investigation of cytotoxic and genotoxic effect of nicotine on human cells

Sandeep Chovatiya*, Sharthak Zaveri and Dipika Patel

Ashok & Rita Patel Institute of Integrated Study and Research in Biotechnology and Allied Science, New Vallabh Vidhyanagar – 388 120, Gujarat.

*Corresponding author email Id: sandeepchovatiya@aribas.edu.in

Abstract:

The objective of the present study was to investigate the potential of nicotine to induce micronucleated polymorphism in tobacco chewing individuals; as well as geneotoxic effect of nicotine on chromosomes. *In vivo* and *in vitro* cytological studies carried out in control and tobacco chewing individuals clearly indicated marked differences. In tobacco chewing individuals, micronuclei (MN) formation was significantly higher along with an increase the size of the nucleus. No significant cell mortality was observed in 10% and 20% nicotine treatment to the leucocytes by MTT assay. The results show that nicotine causes significant genotoxic effect on leukocytes by comate assay and chromosome abbreviation.

Key words: Nicotine, Chewing tobacco, Micronuclei, Cell viability

1. Introduction:

Nicotine is found in a wide variety of plants. However, the principal source of nicotine exposure is through the use of tobacco and nicotine-replacement therapies such as transdermal nicotine patches and nicotine-containing gum [1]. Nicotine is known to have various toxic effects on humans. Also, nicotine is shown to have genotoxic effect on human lymphocytes and tonsillar tissue [2]. Moreover the deaths caused by nicotine are highest in comparison to deaths caused by other factors or diseases as shown in figure-1. The exact effect of nicotine on human cells and DNA is still unknown and so a lot of research still needs to be done in this field.

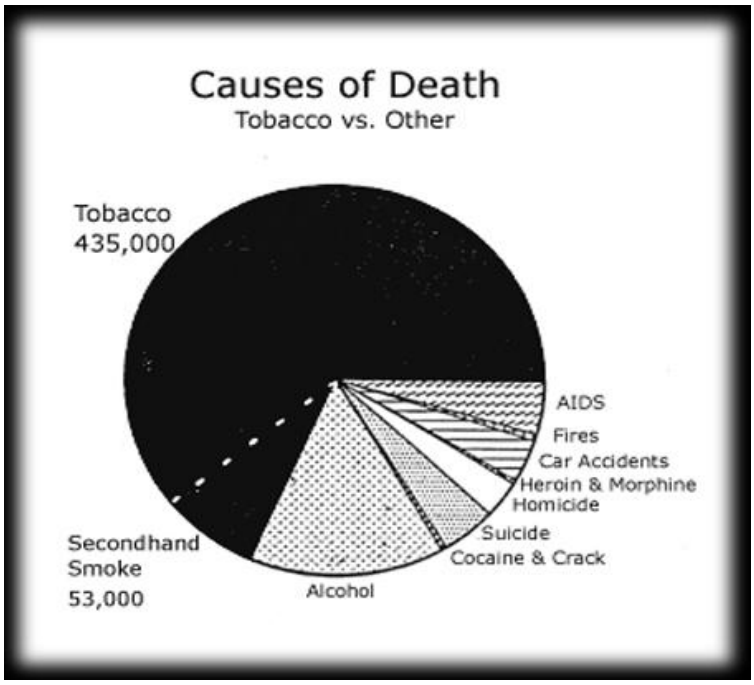

Figure 1: Causes of Death in World Population (The Source is taken from a survey carried out in USA and conducted by WHO).

Barbour *et al.* [3] showed a connection between smoking and Breast Cancer. Tobacco smoke is highly addictive and has been linked to 20 percent of all deaths in the United States. It contains many cancer causing chemicals such as nicotine, and almost one third of all cancer deaths are related to tobacco use. Tobacco smoking has generally been considered to have little or no association with breast cancer risk. Newer studies have challenged this conclusion and suggested a connection between smoking and an increased risk of breast cancer, but more investigation is needed to resolve this issue.

Kleinsasser *et al*. [2] showed that Nicotine demonstrates genotoxicity in human tonsillar tissue and lymphocytes. To assess the genotoxicity of nicotine, the DNA damaging effect on human lymphocytes and target cells from lymphatic tissue of the palatine tonsils from 10 healthy patients was tested with the alkaline single-cell microgel electrophoresis (Comet) assay. The degree of DNA migration, a measure of possible DNA single strand breaks, alkali labile sites, and incomplete excision repair sites, was expressed as the Olive tail moment, the percentage of DNA in the tail, and the tail length. One hour exposure to nicotine at 0.125, 0.25, 0.5, 1, 2,

and 4 mM induced a statistically significant dose-dependent increase of DNA migration up to 3.8-fold and 3.2-fold in tonsillar cells and lymphocytes, respectively.

It is estimated that one in three people in the world will develop cancer at some stage in their lives and that one in four will die from the disease. Smoking is the biggest risk factor for and largest single cause of cancer and approximately one third of all cancer deaths are attributable to smoking. Globally, one in five cancer deaths are caused by tobacco. In October 2009, scientists from 10 countries met at the International Agency for Research on Cancer (IARC) to reassess the carcinogenicity of several compounds, including tobacco. The review, published by The Lancet Oncology, concludes that there is sufficient evidence to confirm that smoking is a cause of 15 types of cancer namely: cancer of the bladder, bone marrow (myeloid leukemia, cervix, colorectal (large bowel), kidney, larynx (voice box), liver, lung, mouth (including lip and tongue), nose, esophagus (gullet), ovaries, pancreas, pharynx (throat) and stomach. The report also states that there is some evidence to suggest that smoking is a cause of breast cancer [4, 5].

About 92% of human cancers are derived from external and internal epithelium. No effective techniques are available for making direct chromosome preparation from epithelium tissues. Casartelli *et al.* [6] observed MN frequencies in exfoliated buccal cells in normal mucosa, precancerous lesions and squamous cell carcinoma. They suggested a link of this bio-marker with neoplastic progressions. The induction in vivo by carcinogens and mutagens is a sign of the genotoxic effect of such substances. The MN assay in exfoliated cells is an innovative genotoxicity technique and holds promise for the study of epithelial carcinogens. It is considered to be a sensitive method for monitoring genetic damage [7].

Direct genotoxic effects of nicotine have been shown in human gingival fibroblasts [8] and spermatozoa [9]. Furthermore, nicotine may also stimulate tumor development by non-genotoxic mechanisms such as angioneogenesis [10, 11], growth stimulation [12], and receptor-regulated cellular growth [13].

The present study reports the cytotoxic and genotoxic effects of chewing tobacco in ex-foliated cells through MN assay; and on lymphocyte through MTT test, cellular apoptosis, COMET assay and chromosomal aberrations through Metaphase plate preparation under in vitro conditions.

2. Materials and Methods

2.1. EXTRACTION OF NICOTINE BY SOXHLET ASSEMBLY:

Tobacco leaves were taken directly from fields. They were then dried and grinded to fine powder and then placed in a thimble made of thick filter paper. The thimble was placed in a soxhlet extractor which was placed onto a flask containing the extraction solvent. The Soxhlet was then equipped with a condenser. The solvent i.e. distilled water was heated to reflux. The cycle was repeated over and over for 6 hours to get a high concentration of the sample in the solvent. Same process was repeated for tobacco obtained commercially (Budhalal, Budhalal & Co. (Tobacco Merchant), Ahmedabad).

2.2. STUDY OF CYTOTOXIC EFFECTS OF CHEWING TOBACCO IN IN-VIVO CONDITIONS:

2.2.1. How the subjects were chosen: The sample was collected from subjects who were known to chew tobacco from last 5-8 years.

2.2.2. Oral smears were obtained from the subjects as follows: The subjects were asked to rinse their mouth thoroughly with water. A pre moistened wooden spatula/toothpick was used to collect the cells from the oral mucosa. The spatula was applied to a pre-cleaned slide to prepare the smear [7].

2.2.3. Staining of cheek cells with Giemsa for identification of Micronulei: Smears were air dried fixed in 80% methanol and stained with Giemsa solution. Cheek cells were observed under 10X and 40X compound microscope for observation of micronuclei [7].

2.3. VIABILITY ASSAY:

The blood cells were cultured in falcon tubes as in leukocyte culturing. After 24 hours of incubation, cells were treated with sample of 10% and 20% nicotine and again incubated for 48 hours. After that cell suspension was taken and 1:1:1 dilution of the suspension was prepared using cells with media, 0.4% trypan blue solution and doubled distilled water. The suspension was loaded on a hematocytometer and immediately observing under microscope.

2.4. MTT TEST:

The MTT assay was used to determine the effect of nicotine on cell growth / proliferation. A 96-well plate was seeded with 100 µl hematocytes along with the media incubated for 24 hours in incubator at 37 °C. After completion of incubation the cells were treated with 10% and 20% nicotine, while control well was exempted from addition of sample and the plate was again incubated at 37 °C for 48 hours. After incubation, plate was removed from incubator and 20 µl MTT reagent solution (1mg/ml; prepared in 1X PBS) was added. The plate was then incubated for 3 hours. After that the 100 µl stop solution i.e. DMSO was added and precipitates were allowed to dissolve for 1 hour followed by spectrophotometric analysis at 490 nm. The results were expressed as percentage of untreated control in RPMI 1640 culture medium.

2.5. DETERMINATION OF CELLULAR APOPTOSIS:

To evaluate whether nicotine induce apoptosis or not, the blood cells were cultured in falcon tubes and sample added and incubated as seen in cell viability assay. After incubation, cells were washed with NH_4Cl at 1500 rpm for 10 minutes until white pellet was obtained. The obtained pellet was suspended in 200 µl saline solution (1X PBS). 100 µl of Hoechst 33342 (1 µg/ml) was added, mixed by inverting 3-4 times and kept in incubator at 37 °C for 40-45 minutes. Then suspension was centrifuged at 1500 rpm for 10 minutes and pellet resuspended in 50 µl of 1X PBS. A drop of the prepared suspension was placed on clean slide, covered by a coverslip and observed under fluorescent microscope.

2.6. COMET ASSAY:

The alkaline version of comet assay was performed according to Singh *et al.* [14] with a slight modification. The culture was prepared and sample added as shown in leukocyte culturing and then incubated for 48 hours. Harvesting was done using 10 ml mixture of 0.85% ammonium chloride (NH_4Cl), 0.1% SDS and a pinch of EDTA (for lyses of RBCs) and centrifuged at 1500 rpm for 10 minutes. Centrifugation was repeated until white pellet was obtained. The white pellet obtained was suspended in 100 µl PBS. Meanwhile, a thin layer of normal melting agarose (1%) was prepared on clean, grease free slides was allowed to solidify. Now on the solidified NMA slides, 20 µl of the obtained white pellet (suspended in PBS) was

added at the centre followed by addition of 5 µl of sample (10% and 20% nicotine). The mixture was properly mixed using a micropipette. Now ~40 µl of low melting agarose (0.5%) was added over the mixture and allowed to solidify. The prepared slides were kept in lysing solution (Stock: 14.6 gm-NaCl, 3.72 gm-EDTA, 0.12-gm Tris HCl, 0.8 gm-NaOH in 100 ml distilled water, pH 10. Working (freshly prepared): 36 ml Stock solution, 4 ml DMSO, 0.4 ml Triton X-100) for 2 hours. After 2 hours the slides were removed from the lysing solution and kept in an electrophoretic unit filled with electrophoresis buffer (3 gm-NaOH, 0.075 gm EDTA in 250 ml D/W, pH >13). The slides were allowed to run for 20 minutes at 50V/300 mA. Immediately the slides were transferred in a neutralizing buffer (4.85 gm-tris buffer in 1000 ml D/W, pH 7.5) for 10 minutes. The slides were lastly stained with 80 µl EtBr (0.1 mg/ml) and left for 5 minutes. Slides were washed with PBS to remove excess stain and observed under florescence microscope.

2.7. LEUKOCYTE CULTURING:

For leukocyte culture, blood was collected from the healthy donor in heparinized tubes. Cultures containing RPMI 1640 (0.8 ml), Blood (0.4ml) 15 % fetal calf serum (1.5ml), PHA (0.1ml), and antibiotics were incubated at 37 °C for 72 h. Then 75 µl of 10% and 20% prepared solution of tobacco extract was added in 4 experimental tubes (2 each respectively) after completion of 24 hours of incubation. Control culture tubes were incubated without tobacco extract. Two hours prior to harvesting, 20 µl EtBr (1 mg/ml) was added to all culture tubes. After that 75 µl colchicine (0.2 mg/ml) was added to culture tubes 1 hour prior to harvesting. For harvesting of cells, the culture was first centrifuged at 1000 rpm for 5 minutes and 8 ml hypotonic solution (560 mg KCl/100 ml) was added to the pellet followed by incubation in water bath at 37 ^{0}C for 35 minutes. After that, 1 ml of chilled fixative (Methanol and Glacial Acetic Acid in 3:1 ratio) was added followed by centrifugation at 1000 rpm for 5 minutes. Supernatant was discarded and again 6-7 ml of chilled fixative was added and centrifuged. This step was repeated twice until white pellet was obtained. The obtained white pellet was suspended in 1 ml of fixative, and slides were prepared by putting few drops of solution on wet slide and were dried followed by staining with 8% Giemsa for 20 minutes [15].

3. RESULTS AND DISCUSSION:

3.1. STUDY OF CYTOTOXIC EFFECTS OF CHEWING TOBACCO IN IN-VIVO CONDITIONS BY OBSERVING THE FORMATION OF MICRONUCLEI:

The results of micronucleus (MN) analysis of oral epithelial cells from person chewing tobacco and, those not chewing tobacco are shown in *figure-2*. All the experimental samples were found to chew different types of tobacco, 4-6 times a day, consuming approximately 2-5 gms of tobacco/day. All of them seem to be taking tobacco since last 5-8 years. Apart from tobacco chewing habit, subjects in experimental samples seem to be not taking any other cytotoxic agents like alcohol, tobacco in smoking form, drug etc.

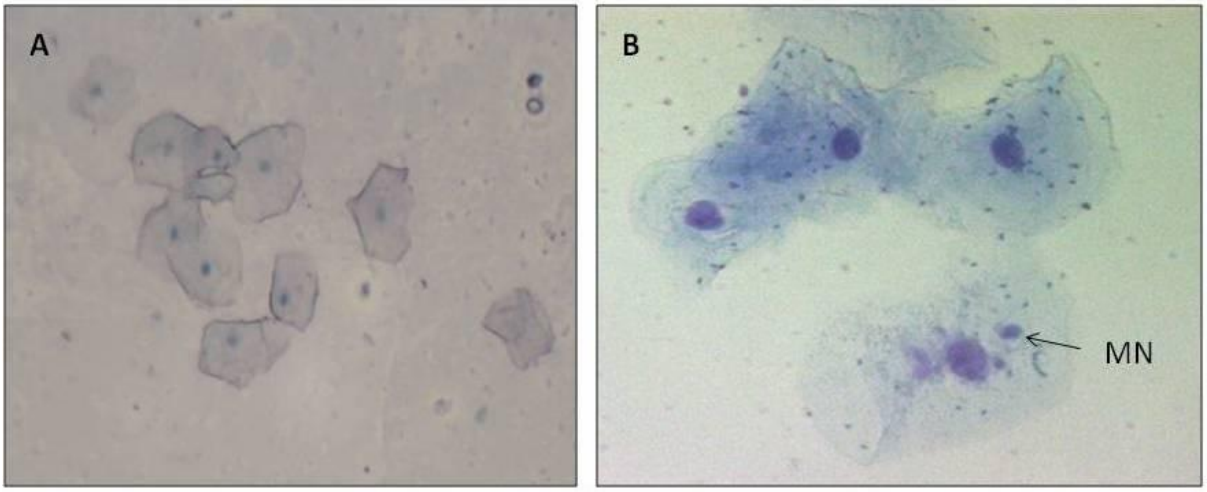

Figure 2: Smear of oral exfoliated cells. Arrow indicates the presence of micronuclei. (A) Control (B) Subject.

Results suggest that controls do not show any micronuclei and so were not taking any cytotoxic agents. Micronucleus assays was performed on oral exfoliated cells of subjects chewing tobacco and not chewing tobacco, using conventional giemsa staining. The experimental subjects showed a significantly higher micronucleus frequency than the control.

Tobacco is a primary cause of cancer. Cigarette smoke is identified as a factor involved in the early stages of tumorogenesis, especially lung cancer and may also be involved in other organs example, Mouth, Pharynx and Bladder [16]. MN methods have been used for diffracting genotoxic effects in human lymphocytes and in oral mucosa cell as well. Halder et al [7] suggested the role of MN frequency as a marker of epithelial carcinogenic progression. According to MN assay is reliable and technically easy to perform and is an efficient alternative to the metaphase analysis. It can also be used as marker of epithelial carcinogenic progression. MN is considered to be the result of chromosome fragments or whole chromosome lagging behind the genome at the cell division. They arise from fragments

produced by chromosome breaks, by certain types of chromatid interchanges and from whole chromosomes. At telophase, they form a small nucleus near the main one from which they are derived.

3.2. CELL VIABILITY:

The viability of cells can be analyzed by staining cells with trypan blue. Live cell exclude dye from entering because of intact cell-membrane and thus remained colourless while the dead cells were stained blue. The result showed that there was no significant effect on cell viability and most of the cells treated with different concentration of sample remained viable. The viability of cell in control was almost 100 %, while it was 99% and 95% in cells treated with 10% and 20% nicotine respectively. There was no observable change in cell morphology.

Kleinsasser *et al*. [2] showed that nicotine did not exert cytotoxic effects in any of the experiments based on the trypan blue test. Cell viability was well above 75% in both cell types before and after exposure to nicotine. In another experiment to study effect of nicotine on cell viability and morphology of human fibroblasts, the test results revealed that more unviable cells were found in the groups exposed to nicotine, in comparison with the control group. Moreover, with increasing doses of nicotine there was a directly proportional increase in the number of unviable cells [17]. It was also shown that Cigarette smoke condensate at 50 lg/mL induced a moderate increase in cell viability, whereas the corresponding nicotine concentration (3.2 lg/mL) did not produce this response. Cigarette smoke condensate at 250 lg/mL, but not nicotine at 16 lg/mL (the corresponding nicotine concentration) induced cell death [18]. To understand the effects of nicotine in human retinal pigment epithelial (ARPE-19), human microvascular endothelial cells (HMVEC) and rat neurosensory retinal (R28) cells, ARPE-19, HMVEC and R28 cell cultures were treated with 10^{-2} and 10^{-4} nicotine for 24 h. Trypan blue dye exclusion assay was performed. With 10^{-2} M nicotine treatment, R28 cell cultures and HMVEC cultures showed decreased cell viability, while ARPE-19 cells showed no change in cell viability [19].

3.3. MTT ASSAY:

The cytotoxic effect of nicotine on cell proliferation of hematocytes was evaluated by using MTT assay. The graph obtained is shown below:

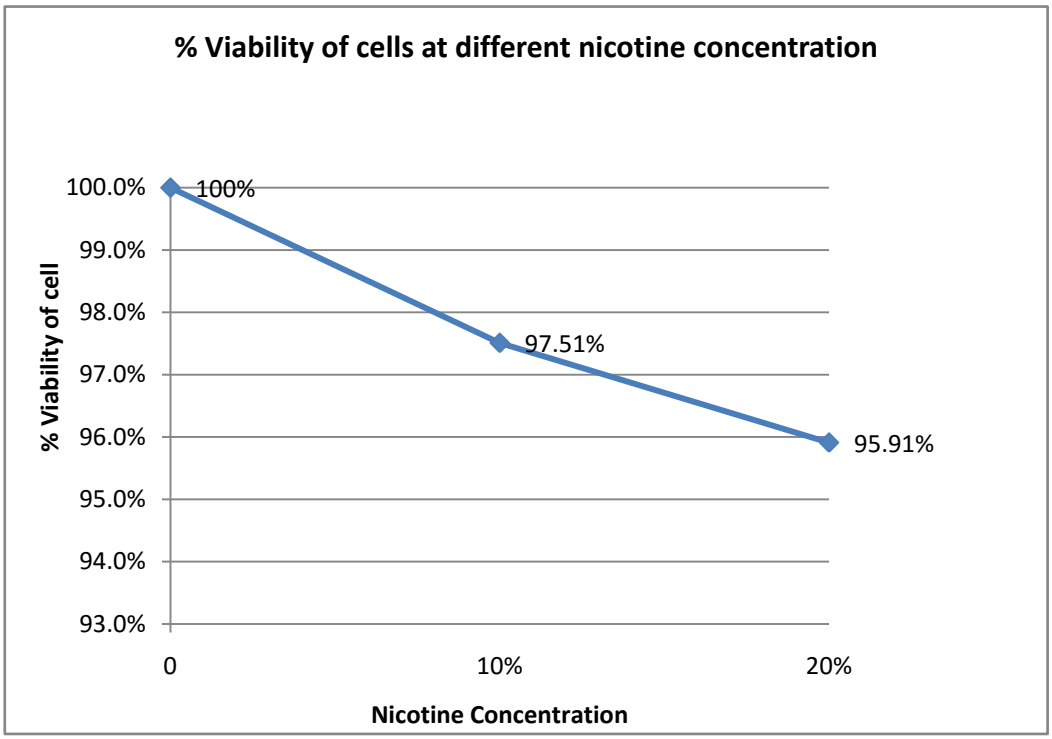

Figure 3: Cell viability at 10% and 20% nicotine concentration by MTT test

The results showed that upon incubation with nicotine for 48 hour, there was a dose-dependent increase in mitochondrial dysfunction within cells. There was a statistically countable difference in cell viability by various concentration of nicotine, compared to the untreated control. Though there was notable decrease in mitochondrial dysfunction in treated cell, the overall viability of cell remain >95%. Nicotine inhibited the proliferation of immortalized and malignant keratinocytes in dose- and time-dependent manners as determined by MTT assay [20]. The significant decrease of MTT reduction and increase of lipid peroxidation in PC12 cells were only observed at treatments with high concentrations of nicotine (1 and 10 mM) [21].

3.4. CELL APOPTOSIS:

The nuclear chromatin of cells was stained with Hoechst 33342 fluorochrome, followed by observation under a fluorescence microscope. Apoptotic cells were distinguished from viable cells by observing the condensed chromatin which was of bright blue colour, while viable cells showed light blue colour. Nicotine showed dose dependent apoptotic effects on lymphocyte cells, compared with untreated cells.

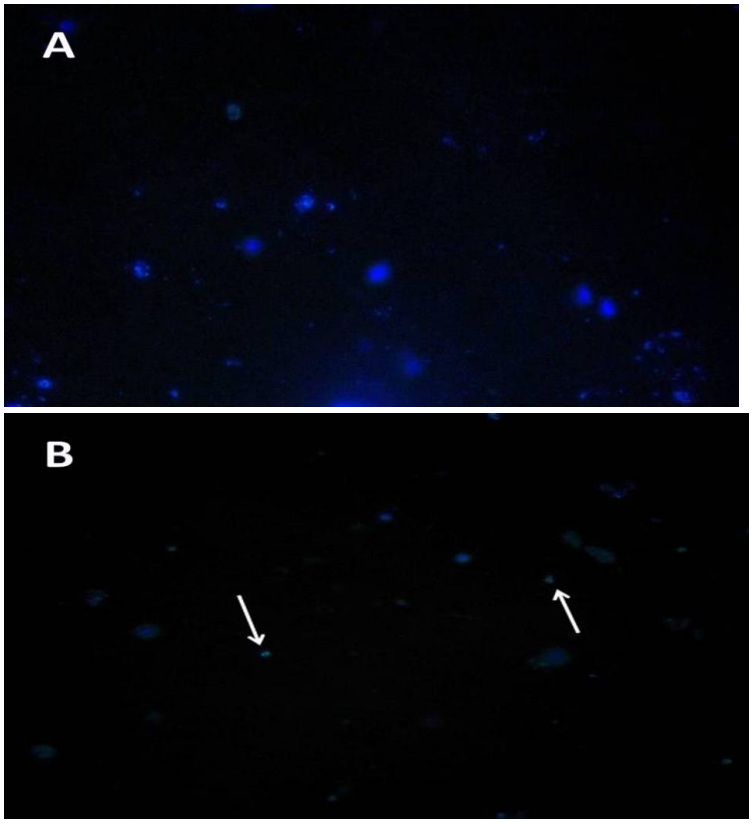

Figure 4: Fluorescent photograph of lymphocyte cells stained with Hoechst 33342. (A)Control (B) 20% nicotine. The arrow indicates the brightly blue stained chromatin of dead cells.

The results indicate that as nicotine concentration increases there is a gradual increase in the no. of apoptotic cells (Figure 4). Control as well as culture with 10% nicotine did not show any apoptotic cell while culture with 20% nicotine showed very few (~1-2) apoptotic cell/s.

Hoechst 33342 is a popular cell-permeant nuclear stain that emits blue fluorescence when bound to dsDNA [22]. Hoechst 33342 induce apoptosis in the HL-60 cells in a time- and dose-dependent manner [23]. Nicotine induces paradoxical effects that might alternatively enforce survival or trigger apoptosis. Nicotine acts as an inducer of apoptosis in normal or transformed lymphocytes, and possibly other non-neuronal cells [24]. When the rats were pretreated by nicotine, neuronal degeneration was attenuated. This observation was confirmed by Hoechst 33342 staining which allowed identifying of cells with condensed, fragmented nuclei, which has been proposed to be a vital sign of apoptosis [25].

3.5. COMET ASSAY:

DNA level damage at every individual cell level can be measured by the comet assay.

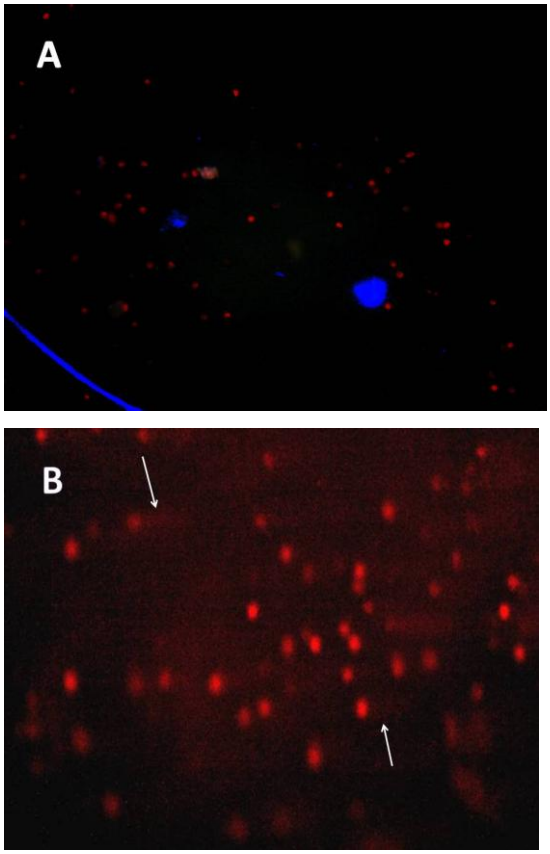

Figure 5: Effect of nicotine and formation of comet tail (A) Control-20X (B) Cells treated with 20% nicotine-45X

The results show that nicotine causes significant genotoxic effect on leukocytes. The DNA damage can be visualized as the length of comet tail formed (*Figure 5*). The alkaline single-cell microgel electrophoresis (Comet) assay is a sensitive tool for detection of DNA single strand breaks, as well as foralkali labile and incomplete excision repair sites [26]. Lymphocytes and mucosal cells as human target cells have been analyzed by this method [27]. Kleinsasser *et al*., [2] showed that 1 hr of incubation with nicotine induced a significant concentration-dependent increase in DNA migration in the Comet assay in tonsillar cells as well as in peripheral lymphocytes from the same donors. When highly pure nicotine from two different commercial sources was used, the significant concentration dependent DNA damage by nicotine was confirmed for both tonsillar cells and peripheral lymphocytes. There was no difference in DNA migration as far as the source of nicotine was concerned. A similar effect was shown previously for myosmine, a minor tobacco alkaloid that also occurs in a variety of foods, e.g., cereals, nuts, cocoa, and dairy products [2, 28]. Nicotine was equally genotoxic to lymphocytes and tonsillar cells. This finding is in contrast to results obtained for other

genotoxic compounds such as vanadium and, to a lesser extent, phthalates and myosmine, which were more genotoxic to lymphocytes than to mucosal cells [2]. An analysis of a possible influence of smoking status on DNA migration in the negative controls showed no significant differences. This finding is in line with results of Hoffmann and Speit [29], showing no differences in peripheral blood cells from heavy smokers and nonsmokers, in the comet assay. A significant increase was observed in Olive tail moment and tail length was obtained when dose of nicotine was increased. Higher dose of nicotine showed significant DNA damage while at lower conc. no damage was detected by comet assay [30].

3.6. LEUKOCYTE CULTURING:

Leukocyte culture in the presence of nicotin has exhibited different chromosomal aberrations like ring chromosome, chromosome breaks and with total change in chromosomal organization. *Figure-6* shows that different chromosomal aberrations are observed. The results found in tubes with 20% nicotine sample added to it, shows the formation of ring chromosome as shown in *Figure 6 (B),* while chromosome condensation was observed *Figure 6 (C)* in another culture.

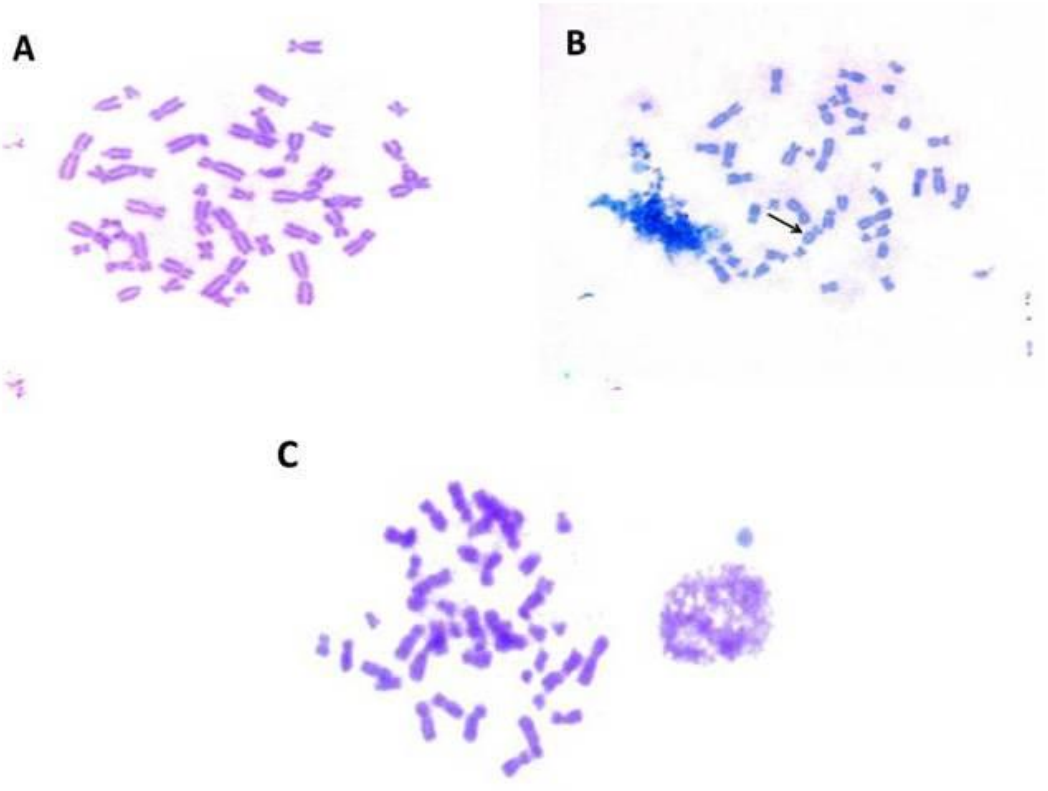

Figure 6: Metaphase plate showing chromosomes.(A) Control (B) & (C) Effect on chromosome by treatment with 20% nicotine. (B) Ring Chromosome - Arrow shows the occurance of ring chromosome (C) Completely Condensed Chromosome

Development of chromosomal aberrations in cultured leukocytes in the presence of nicotine confirms the genotoxic effect of nicotine most probably by forming DNA adducts. Though we could only check the effects of nicotine at the concentration of 10% & 20% only, the effects of nicotine with different concentrations should further be studied for the proper evaluation of genotoxic effect. When leukocytes were cultured with tobacco extract, chromosomal aberrations like chromatid break, dicentric chromosomes, chromosome ring formation and complete change in the organization could be observed. In a similar observation, elevated chromosomal aberration frequencies in smokers have been reported [31, 32]. The review, published by The Lancet Oncology, concludes that there is sufficient evidence to confirm that smoking is a cause of 15 types of cancer [5]. Tobacco metabolites formed in the form of carcinogens form DNA adducts, usually with adenine or guanine. DNA adducts which escape cellular repair could persist and may lead to miscoding and mutation [33].

4. CONCLUSION:

The findings suggest that micronuclei assay of oral epithelial cells can be used as simple markers for understanding genotoxic damage caused by chewing tobacco products by people and they can be warned for possible development of cancer later in life. We could also confirm the genotoxic effects of tobacco in *in-vitro* conditions on cultured leukocytes by studying chromosomal aberrations and by performing comet assay. The formation of comet tail on cultured leukocytes indicates the DNA damage done by nicotine. Though there is no significant change in cell viability or cell apoptosis we could conclude that nicotine causes a dose-dependent damage on human hematocytes. As the concentration of nicotine increases there is a gradual decrease in viable cells and an increase in apoptotic cells. The exact genotoxic and cytotoxic effect of nicotine is still not known and thus a further research needs to be focused in this field.

5. ACKNOWLEDGEMENTS

The author would like to thanks Dr. Joshi and Dr. Rank, Veterinary Dept. Anand Agriculture University, Anand, Gujarat for provides the microscopy facility. The author would also like to thank SICART, Vallabh Vidhyanagar, Gujarat for instrumental facility.

6. REFERENCES:

[1] Heisheman, S.C., Taylor, R.C. and Henningfield, J.E. Nicotine and smoking: a review of effects on human performance, Exp. Clin. Pharmacol. (1994), 2: 345–395.

[2] Kleinsasser NH *et al.* - Toxicological Sciences, (2005), 86(2), 309–317.

[3] Barbour S. Warren et al. - Cornell University Program on Breast Cancer and Environmental Risk Factors in New York State (BCERF) (2002).

[4] Peto R et al. Mortality from smoking in developed countries 1950-2000 (2nd edition) Oxford University Press (2004).

[5] Straif Kurt et al. The Lancet Oncology, (2009), 10 (11), 1033 – 1034.

[6] Casartelli G, Bonatti S, De Ferrari M, Scala M, Mereu P, Margarino G, Abbondandolo A. Micronucleus frequencies in exfoliated buccal cells in normal mucosa, precancerous lesions and squamous cell carcinoma. Anal Quant Cytol Histol. (2000), 22(6):486-92.

[7] Halder A., T. Chakraborty, K. Mandal, P. K. Gure, S. Das, R. Raychowdhury and A. K. Ghosh Comparative Study of Exfoliated Oral Mucosal Cell Micronuclei frequency in Normal, Precancerous and Malignant Epithelium. Int J Hum Genet, (2004), 4(4), 257-260.

[8] Argentin, G., and Cicchetti, R. Genotoxic and antiapoptotic effect of nicotine on human gingival fibroblasts. Toxicol. Sci. (2004), 79, 75–81.

[9] Arabi, M. Nicotinic infertility: assessing DNA and plasma membrane integrity of human spermatozoa. Andrologia, (2004), 36, 305–310.

[10] Heeschen, C., Jang, J. J., Weis, M., Pathak, A., Kaji, S., Hu, R. S., Tsao, P. S., Johnson, F. L., and Cooke, J. P. Nicotine stimulates angiogenesis and promotes tumor growth and atherosclerosis. Nat. Med. (2001), 7, 835–841.

[11] Cooke, J. P., and Bitterman, H. Nicotine and angiogenesis: A new paradigm for tobacco-related diseases. Ann. Med. (2004), 36, 33–40.

[12] Shin, V. Y.,Wu,W. K. K., Ye, Y.-N., So,W. H. L., Koo, M.W. L., Liu, E. S. L., Luo, J.-C., and Cho, C.-H. Nicotine promotes gastric tumor growth and neovascularization by

activating extracellular signal-regulated kinase and cyclooxygenase-2. Carcinogenesis, (2004), 25, 2487–2495.

[13] Schuller, H. M., Plummer, H. K., and Jull, B. A. Receptor-mediated effects of nicotine and its nitrosated derivative NNK on pulmonary neuroendocrine cells. Anat. Rec., (2003), 270A, 51–58.

[14] Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Experimental Cell Research. (1988) 175(1):184–191.

[15] Patel R. K., Radhakrishna U. and Khoda V. K.. A modified technique of GTG banding in Cattle and buffaloes. The Nucleus. (1995), 38: 37-39.

[16] Rowland RE, Harding KM. Increased sister chromatid exchange in the peripheral blood lymphocytes of young women who smoke cigarettes. Hereditas, (1999), 131(2):143-6.

[17] Martinez AE, Silverio KG, Rossa C Jr. - Effect of nicotine on the viability and morphology of fibroblasts: in vitro study. Brazilian Oral Research. (2002), 16(3):234-8.

[18] Silva D, Cáceres M, Arancibia R, Martínez C, Martínez J, Smith PC. Effects of cigarette smoke and nicotine on cell viability, migration and myofibroblastic differentiation. Journal of Periodontal Res. (2012), 47(5):599-607.

[19] Patil AJ, Gramajo AL, Sharma A, Seigel GM, Kuppermann BD, Kenney MC. Differential effects of nicotine on retinal and vascular cells in vitro. Toxicology. (2009), 259(1-2):69-76.

[20] Lee HJ, Guo HY, Lee SK, Jeon BH, Jun CD, Lee SK, Park MH, Kim EC. Effects of nicotine on proliferation, cell cycle, and differentiation in immortalized and malignant oral keratinocytes. Journal of Oral Pathology and Medicine, (2005), 34(7): 436-43.

[21] Guan ZZ, Wen-Feng Yu, Agneta Nordberg (2003) - Dual effects of nicotine on oxidative stress and neuroprotection in PC12 cells. Neurochemistry international. Volume 43, Issue 3, August 2003, Pages 243–249.

[22] Oriowo OM, Cullen AP, Sivak JG. Impairment of eye lens cell physiology and optics by broadband ultraviolet A-ultraviolet B radiation. Photochem Photobiol. (2002), 76:361–7.

[23] Zhang X, Chen J, Davis B, Kiechle F. Hoechst 33342 induces apoptosis in HL-60 cells and inhibits topoisomerase I in vivo. Arch Pathol Lab Med. (1999), 123(10):921-7.

[24] Oloris SC, Ashley A. Frazer-Abel, Cristan M. Jubala, Susan P. Fosmire, Karen M. Helm, Sally R. Robinson, Derek M. Korpela, Megan M. Duckett, Shairaz Baksh and Jaime F. Modiano. Nicotine-Mediated Signals Modulate Cell Death And Survival Of T Lymphocytes. Toxicology and Applied Pharmacology. (2010), 242(3):299-309.

[25] Riljak V., M. Milotová, K. Jandová, J. Pokorný, M. Langmeier. Morphological changes in the Hippocampus following nicotine and kainic acid administration. Physiol. Res. (2007), 56: 641-649.

[26] Lee E., Lee J., Sul D., and Lee J. Use of the tail moment of the lymphocytes to evaluate DNA damage in human biomonitoring studies. Toxicol. Sci. (2004), 81, 121–132.

[27] Harrus U., Schmezer P., Kuchenmeister F. and Maier H. Genotoxic effect on human mucous membrane biopsies of the upper aerodigestive tract. Laryngorhinootologie, (1999), 78, 176–181.

[28] Tyroller S., Zwickenpflug W., and Richter E. New sources of dietary myosmine uptake from cereals, fruits, vegetables and milk. J. Agric. Food. Chem. (2002), 50, 4909–4915.

[29] Hoffmann H., and Speit G. Assessment of DNA damage in peripheral blood of heavy smokers with the Comet assay and the micronucleus test. Mutat. Res., (2005), 581, 105–114.

[30] Sobkowiak R, Lesicki A. Genotoxicity of nicotine in cell culture of Caenorhabditis elegans evaluated by the comet assay. Drug Chem Toxicol. (2009), 32(3):252-7.

[31] Obe G., Herha, J. Chromosomal aberrations in heavy smokers, Hum. Genet. (1987), 41, 259-263.

[31] Vijayalaxmi, H. J. Evans. In vivo and in vitro effects of sister chromatid exchanges in human peripheral blood lymphocytes, Mutat. Res. 92 (1982) 321-332.

[33] Kuper H, Adami HO, Boffetta P. Tobacco use, cancer causation and public health impact. J Intern Med. (2002), 251(6):455-66.