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Micronucleus Assessment in Human Buccal Cell DNA As a Contrivance for Bio-Surveilling DNA Impairment

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Abstract

Introduction: Oral cancer is one of the most common causes of transience in India. A lack of definitive and adequate biological markers for early oral cancer detection, combined with a scarcity of definitive and suitable biological markers, has resulted in delayed stage diagnosis of oral carcinoma. A buccal cell could be a biomarker for cancer. For monitoring genetic deterioration in people, micronucleus evaluation in exfoliated buccal cells is a useful and least intrusive procedure.

Aim and objective: To assess the DNA impairment in tobacco related human buccal cells using micronucleus assessment.

Study design: The study included 49 participants who were divided into three groups based on their tobacco use. Group I consisted of 15 people who had never have tobacco usage, Group II included 19 people who had the habit of tobacco usage, and Group III of 15 people who had oral carcinoma associated with tobacco habits. Buccal cells from these people were utilized to look for tobacco-related DNA damage using a PCR approach that measured tail length.

Results: Between all groups, the P value for micro nucleated cells of buccal mucosa was 0.001, which was statistically significant. The midpoint tail length was found to be 1.46 µm in the normal mucosa, 2.86 µm in tobacco users, and 3.86 µm in tobacco users' oral cancer. Age, gender, length, and different types of tobacco use all had an impact on the oral mucosa.

Conclusion: The micronucleus assay can help detect subclinical genetic changes in the oral mucosa before they appear clinically as precancerous lesions owing to tobacco use. In the near future, micronucleus study may emerge as a novel adjuvant tool for the prevention of oral malignancy.

Keywords: Micronucleus assessment, PCR assay, DNA impairment, tobacco

Introduction

India is thought to be the global capital for oral cancer cases, accounting for one-third of the global burden. The southern portions of India have the highest prevalence rate of oral cancer. The two key factors that influence the incidence of oral cancer are genetic and epigenetic. Tobacco's purpose of causing chaos and death has a significant impact on public health. The early detection of potentially malignant illnesses not only reduces the risk of oral cancer, but also improves the chances of survival [1-3]. Many carcinogens target DNA (deoxyribonucleic acid), which is one of their primary targets. DNA is commonly utilized for PCR-based diagnostics white cells fractionated from whole blood were used to make the assessment. Although this method provides large amounts of DNA, it has significant drawbacks, such as the interruption of collecting blood, the potential of exposure to blood-borne pathogens, liquid sample handling, and a lengthy extraction procedure. Hair roots, cheek scrapings, and buccal cell samples have also been used to acquire DNA for genetic diagnosis. A growing range of

systemic diseases and disorders, including oral cancer, have been discovered to be diagnostically reflected in saliva. Oral saline rinses have been extensively utilized to harvest buccal epithelial cells for DNA extraction. Because carcinogens come into direct touch with buccal epithelial cells during tobacco use, detecting DNA damage in these cells would be advantageous and suitable.

Furthermore, using saliva as a diagnostic fluid satisfies the desire for cost-effective and easily accessible diagnostic methods[4]. Buccal cells in saliva are a good source of DNA since they can be acquired using a self-administered and reasonably noninvasive approach[5-11]. Sample collection for DNA extraction is an important technique because it takes time and may involve ethical issues. As a result, it is critical that this technique become simpler, less expensive, and free of the use of phenol-chloroform, as in other procedures.

Methodology

Preliminary study

Study population

A total of 49 volunteers, both male and female, ranging in age from 20 to 54 years, were enrolled in the study to harvest buccal cells using a mouthwash solution. Everyone who took part in the study gave their informed consent. Participants were provided data collection papers and asked to take buccal cell samples after providing signed informed consent[12-15]. The sample size formula (n) = 4pq/L2, where p is the expected proportion of samples revealing micronuclei in percentage, q is (1-p), and L is the allowable error in estimating p, was used to collect data. On the basis of tobacco use, the 49 subjects in the study were divided into three categories:

- Category I (Control group): 15 healthy participants who did not use tobacco and did not have an oral lesion.
- Category II: 19 participants had tobacco and related habits but no habit-related oral lesion.
- Category III: 15 participants who used cigarettes and had other tobacco-related habits, as well as oral cancer.

The study included patients in the categories I, II, and III who had oral lesions on clinical evaluation, based on the following inclusion and exclusion criteria:

Inclusion criteria

- Subjects who have used tobacco and associated products for at least 5 years.
- People who have tobacco-related oral lesions or oral cancer.

Exclusion criteria

- Subjects having oral lesions other than tobacco associated oral lesions.
- Subjects who have already been medicated for tobacco-related oral lesions.
- Subjects with any type of systemic illness.

- Subjects who have had any type of radiography procedure during the last month.
- Those that have been exposed to a DNA-damaging substance or ionizing radiation.

Sample collection

The participant was approached with a sterile leak proof cup with lid as a collecting container containing the solution to be swished on the inside of their mouth the day before in the evening. Mouthwash was swished for 60 seconds and spit into a sterile receptacle in groups [16-18]. Participants were asked to stop smoking, drinking, or eating 45 minutes before the sample collection.

Sample processing

Prior to the extraction stage, the acquired sample was stored at room temperature [19].

Isolation of DNA

Centrifugation at 10,000 rpm for 10 minutes at room temperature yielded the buccal epithelial cells, and the supernatant was discarded. The pellet was transported to a screw-capped microcentrifuge after being resuspended in 500 µl of 10 mM NaCl and 10 mM EDTA (pH 7.5). The supernatant was discarded after centrifuging the solution for 15 seconds. By vortexing for 10 seconds, the cell pellet was resuspended in 500 µl of 50 mM NaOH. For 5 minutes, the sample was incubated at 100°C. The content was neutralized by vortexes for 5 seconds in 100 µl of 1 M Tris-HCl [20-23]. The samples were also centrifuged for 15 seconds to eliminate cell debris while keeping the supernatant.

Assessment of DNA quality

Using agarose gel electrophoresis, the extracted DNA was examined. A 2 percent agarose gel was loaded with 3 µl of DNA [24-29]. Bromophenol blue dye was used to stain nucleic acids. The ethidium bromide dye was utilized as both a control and a marker for high-quality DNA. The voltage was supplied at a rate of 1-5 V/cm. When the DNA samples had travelled a sufficient distance through the gel, the electric current was turned off, and the lids of the gel tank were removed [30-38]. Under UV light, the gel was examined. DNA standards were recorded according on molecular mass, humiliation level, and staining intensity.

DNA Probity

Isolated DNA was electrophoresis on a 0.4 percent agarose gel at 2V/cm in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.3) and stained with ethidium bromide to determine the presence of high molecular mass DNA in buccal cell samples. Disintegration of buccal cell DNA samples against a known molecular mass marker (Ready-load DNA/HindIII disintegration; Life Technologies, Inc.) with visible bands of lengths of 23 bp, 130 bp, 9,416 bp, 6,557 bp, 4,361 bp, 2,322 bp, 2,027 bp, and 564 bp was used to check for DNA humiliation[39-42].

PCR assessment

A PCR test was used to measure DNA probity. DNA multiplication is an *in-vitro* nucleic acid multiplication process that uses Taq DNA polymerase and at least two oligonucleotides that are identical to the DNA to be multiplied. The primers used for inspection was

Forward primer IFN 3F:5'GGCACAACAGGTAGTAGGCG3' and
Reverse primer IFN5R:5'5'GCCACAGGAGCTTCTGACAC3'.

In a 500 µl PCR tube, the reagents were added in the following order: 38 µl of sterile MilliQ water was added in a 0.2/0.5 ml microfuge tube. 1 µl of sample DNA (200 ng/ml), followed by 5 µl of 10X Taq polymerase evaluation buffer with $MgCl_2$ and 3 µl of 10 mM dNTP mix (2.5 mM each) solution, 2 units of Taq DNA polymerase (3 units /µl) and 1 µl each of forward and reverse primers (250 ng/µl) were also added [43-44].

PCR initiative

Denaturation for 9 minutes at 94°C, for 35 cycles. Annealing at 55°C for 1 minute, extend at 72°C. Finish with a 10 minute extension at 72°C. As a negative control, a reaction mixture without DNA was used. The RAPD-PCR was carried out in an Eppendorf thermal cycler with the lid preheated to 102°C, which prevented the accumulation of paraffin layer and evaporation owing to temperature differences [45]. Following the reaction, 10 µl of aqueous layer was run on a 1 percent agarose gel stained with ethidium bromide for 1 to 2 hours at 100 volts and visualized under UV illumination for the required length of DNA.

PCR-RFLP (Restriction fragment length polymorphism)

For the polymorphism, DNA samples from all three groups of buccal cell collection were tested using the PCR-RFLP methodology. (Restriction enzyme 2-3 units – 0.5 µl, Enzyme buffer 10X –1.0 µl, and DNA sample –1.0 µl) make up the reaction mix. It was incubated for 1 hour at the recommended temperature. At -20 ^{0}C, 2.5 µl 6 X loading dye mix was added to block the reaction [45]. The digested DNA was put onto an agarose gel and ran at 100 V for 1 to 2 hours, with DNA fragments being seen under UV and compared to a DNA marker.

Statistical analysis

The ANOVA test was used to compare the three group samples to see if there were any changes in mean total DNA yield and PCR quantification under different conditions. For the average number of micronuclei in cells and micro nucleated cells of the buccal mucosa, a paired t-test was used. Control variables that differentiate between habit with oral lesions and habit without oral lesions were examined using discriminate function examination. In all statistical analyses, $P< 0.05$ was found to be statistically significant.

Results

DNA was successfully retrieved from saliva samples throughout our protocol. There was a wide range of DNA yields identified. A spectrophotometer was used to record the concentration and purity of the DNA recovered from the saliva samples.

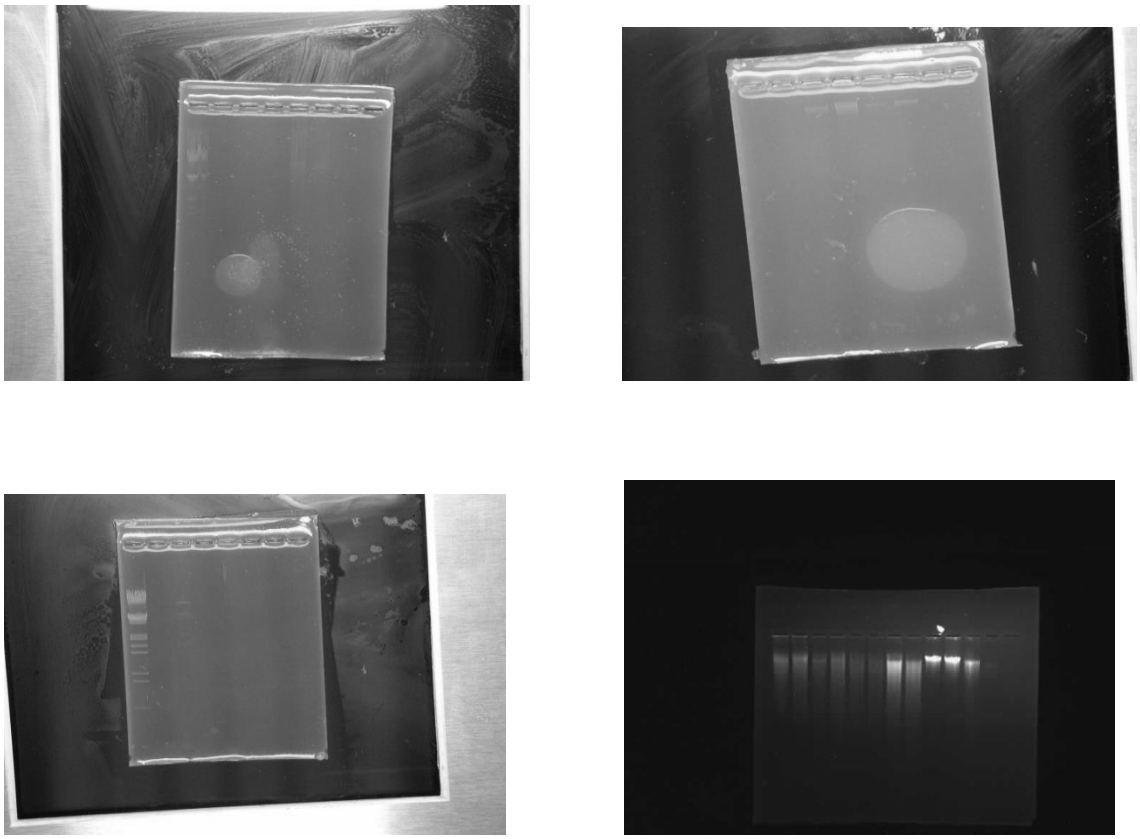

Figure 1: Photos of agarose gels graph of normal, tobacco user, and tobacco user with oral cancer comparison study.

The end result shows a high yield of pure DNA. At 260 nm, the total DNA yield was 18.27.3 µg (10.1 - 37.8 µg), 22.214.99 µg (9.1 - 52.5 µg), and 23.911.8 µg (9.2 - 43.4 µg), respectively, and the mean OD 260/280 ratio was 2.010.04 (1.96-2.13), 4.010.04 (1.94-2.03), and 5.010.09 (1.87-2.15) for Group I, II, and III The presence of high-molecular-weight DNA was visualized using gel electrophoresis. This result suggested that there was a significant difference in DNA yield (p=0.71) and purity (p=0.82) between the three groups. The non-degradation is supported by the gel electrophoresis data. Furthermore, the isolated DNA of the samples was successfully PCRable, showing that the overall amount of human DNA is sufficient and that the DNA samples are not excessively damaged.

The collected DNA was then tested for quality using PCR, and the results showed that in the majority of cases with the three groups, the isolated DNA could be amplified for short fragments. The synthesized primers were subjected to more gradient PCR, with the annealing temperature set at 55 °C, yielding a single, conspicuous band of 134 bp. For some DNA samples, the PCR-RFLP processes were successfully preceded. Three different groups' shows PCR products by restriction enzymes are illustrated in Figure 2.

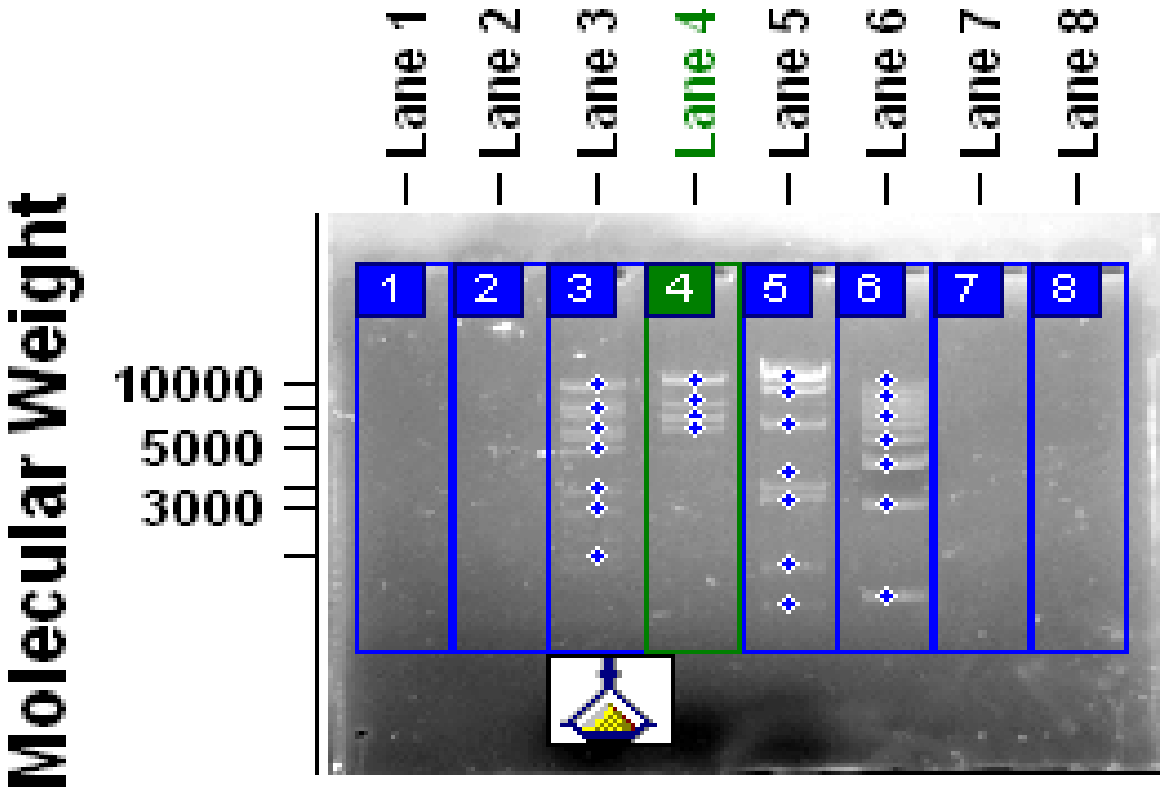

Figure 2: PCR-RFLP-reaction of three groups

Group I

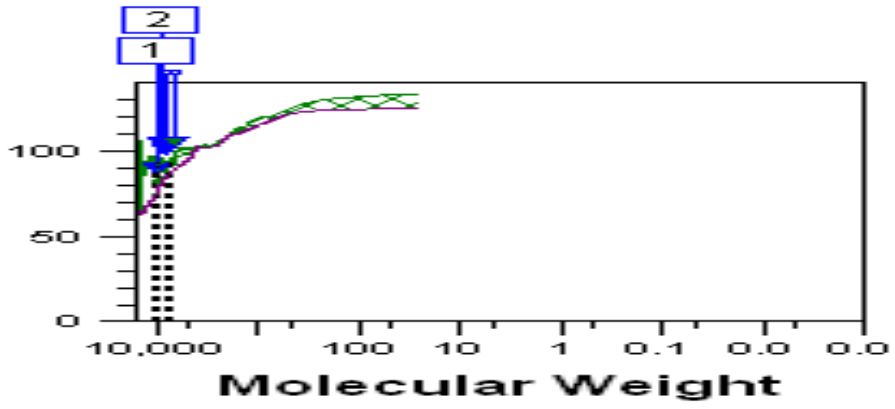

Figure 3: Molecular weight of healthy people DNA

Band	Posn	Volume	Vol+BkGnd	Calib Vol (ng)	Area Lane %	MW	Rf
1	99	5254.95	47943.00	576.00	2.33	10263.16	0.30

2	118	11337.34	99370.00	1080.00	5.03	9334.74	0.36
3	131	646.81	18860.00	216.00	0.29	8291.61	0.40
4	142	36006.46	1512862.00	3168.00	15.98	7000.00	0.43

Group II

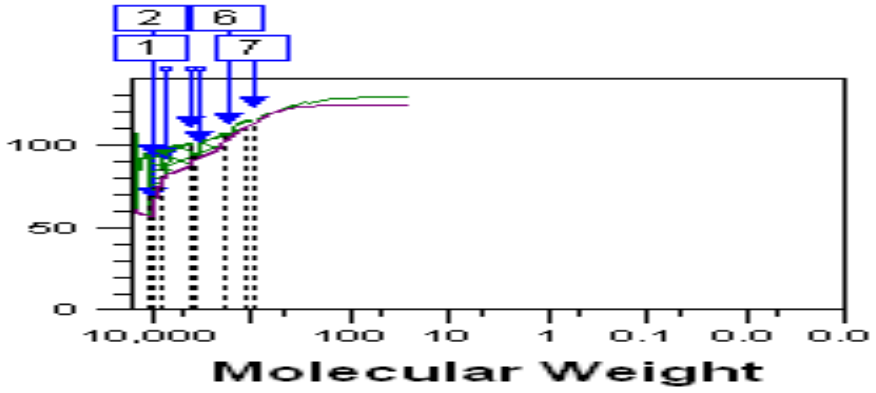

Figure 4: Molecular weight of tobacco chewing habit people DNA

Band	Posn	Volume	Vol+BkGnd	Calib Vol(ng)	AreaLane %	MW	Rf
1	96	4100.34	32667.00	504.00	1.25	10421.05	0.29
2	112	38951.12	174933.00	1944.00	11.92	9652.57	0.34
3	139	35872.99	277395.00	2880.00	10.98	7385.21	0.42
4	180	11961.17	120600.00	1224.00	3.66	4236.70	0.54
5	202	26501.42	419195.00	4104.00	8.11	3508.58	0.61
6	259	8136.84	259528.00	2376.00	2.49	1775.00	0.78
7	291	1005.74	49184.00	432.00	0.31	975.00	0.88

Group III

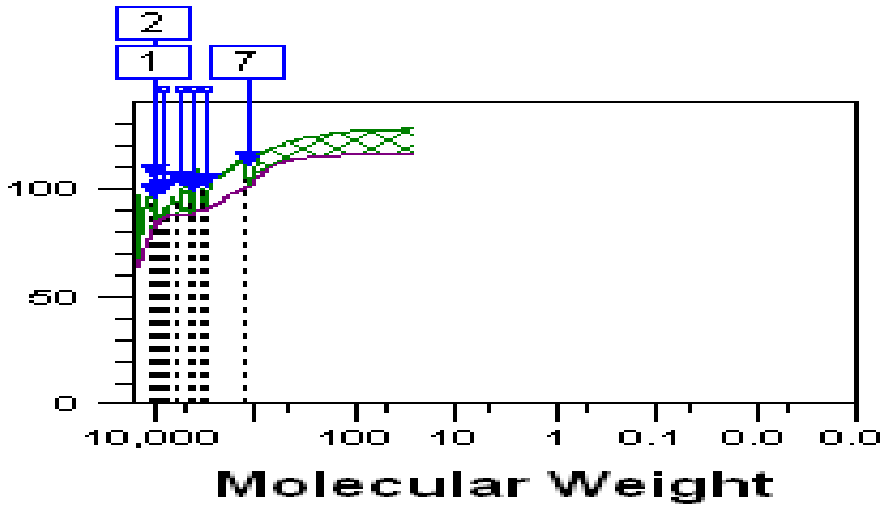

Figure 5: Molecular weight of tobacco chewing habit with oral cancer people DNA

Band	Posn	Volume	Vol+BkGnd	Calib Vol(ng)	AreaLane %	MW	Rf
1	99	11569.50	112099.00	1224.00	4.34	10263.16	0.30
2	115	599.00	42817.00	504.00	0.22	9502.86	0.35
3	131	1521.00	57546.00	648.00	0.57	8291.61	0.40
4	153	13467.00	133091.00	1368.00	5.05	5536.02	0.46
5	172	1853.65	45931.00	504.00	0.69	4322.30	0.52
6	207	2005.20	40770.00	432.00	0.75	3183.76	0.63
7	286	28890.96	430202.00	3672.00	10.83	1100.00	0.86

In the semi quantitative analysis, there was a significant difference between the control samples and the tobacco-related oral cancer samples. The frequency of micronuclei was substantially higher in the malignant disease group than in the control group (p value < 0.001).

CONCLUSION

Tobacco use is one of the most important etiological agents, as it leads to a variety of oral lesions with a higher risk of malignant transformation. When a DNA repair enzyme fails to manage DNA damage, uncontrolled proliferation and reduced apoptosis occur. Early detection and examination of possibly malignant tumors will aid in the prevention of cancer at an earlier stage. To detect and inspect these situations, a variety of modern molecular protocols are available. Micronucleus count and apoptosis can be utilized as a functional biomarker and as a screening test for patients who smoke and patients who have oral lesion symptoms, including premalignant lesions The DNA damage in buccal cells was examined as a non-invasive simple methodology in this work, which is critical in the early diagnosis of mouth cancer. For determining abnormalities such as DNA damage, the technique is well-standardized. This noninvasive approach has the potential to persuade people to stop smoking before any evident clinical effects arise. As a result, in the not-too-distant future, micronucleus assay may emerge as a revolutionary array for preventing oral cancer. Finally, while the current protocol is not yet ready for direct clinical use as diagnostic tools, it is clear that a simple, rapid, portable, and cost-effective clinical diagnostic procedure could be available in the near future.

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