CYTOGENETIC BIO MONITORING USING MICRONUCLEI ASSAY IN PETROL PUMP WORKERS USING RAPID PAP STAINING TECHNIQUE-AN OBSERVATIONAL STUDY

Vishnuvarthan Aparna[1]*, Philip Sachu[2], Thukanaickenpalayam Ragunathan Yoithapprabhunath[3], Abraham Philips[4], Arumugam Aarthi[5]

Abstract

The screening and monitoring the petroleum related cytotoxicity in petrol pump workers can be a useful tool to detect initial cellular damage. Aim of our study is to assess the micronuclei frequency in buccal epithelial cells of petrol pump workers. In 80 exposed petrol pump workers, micronuclei frequency (MN) was assessed using PAP stain and compared with unexposed workers of age21-60 years. Statistical analysis was done using SPSS version 25.0. The Mann whitney U test was used to compare the mean MNC between the control and exposed group.MN of exposed population with habits of smoking and tobacco chewing showed significantly high frequency than the exposed group without habits. The MN was significantly higher in workers with longer duration of exposure .The micronuclei assay of exposed petrol pump workers paves a simple and easy method to identify patients with early genomic damage.

Keywords: Micronuclei frequency, genotoxicity, buccal mucosa, PAP stain

[1]*Intern, Vivekanandha Dental college for Women, Tiruchengode, Tamil Nadu, India, 637205, E-mail:- aparnavvarthan@gmail.com[1]

[2]Department OF Biochemistry, Swamy Vivekanandha Medical college Hospitals& RI, Tiruchengode, Tamil Nadu,India,637205, E-mail:- philipsachu1@gmail.com[2]

[3]Professor, Department of Oral Pathology, Vivekanandha Dental College for women, Tiruchengode, Tamilnadu E-mail:- yoitha.dentist@gmail.com3

[4]Professor, Department OF Biochemistry, AL Azhar Medical college & Hospitals, Ezhanallor, Thodupuzha, Kerala, India, , dr.philips@gmail.com[4],

[5]Senior Resident, Department of Oral Pathology, Vivekanandha Dental College for Women, Tiruchengode, Tamilnadu. Email- aarthiarumugam@gmail.com[5]

***Corresponding Author: -** Vishnuvarthan Aparna

*Intern, Vivekanandha Dental college for Women, Tiruchengode, Tamil Nadu, India, 637205, E-mail:- aparnavvarthan@gmail.com

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I.INTRODUCTION

Exposure to petroleum products and the chemical substances released into the atmosphere causes occupational health hazards to the front-line workers of petrol pump and gas stations [1] . The increased growth of the global population and increase in the automobile sector has escalated the need for petrol, demonstrating an increased exposure of the occupationally-exposed work-forces. Genetic damage is the most important basic cause of developmental and degenerative disease. It is also well established that genomic damage is produced by environmental exposure to genotoxins, medical procedures (eg, radiation and chemicals), micronutrient deficiency (eg, foliate), lifestyle factors (eg, alcohol, smoking, drugs, and stress), and genetic factors, such as inherited defects in DNA damage [2].

The presence of micronuclei in exfoliated buccal epithelial cells has proven a useful biomarker of occupational exposure to petroleum products.

Micronucleus (MN) assay for exfoliated cells in buccal cells is more reliable indicator for evaluating the genotoxic effects produced by low doses of carcinogenic substances to which human populations are exposed since buccal mucosa cells are the first to be in direct contact after exposure [3]. The frequency of MN in human exfoliated cells can be used as an "endogenous dosimeter" in tissues that are specific targets of genotoxic and carcinogenic agents, when carcinomas will develop. Petrol evaporates more readily in tropical than intemperate countries. In South India petrol stations are located on streets and workers at the station, have a greater chance for exposure. Petrol bunk workers are engaged in petrol filling for eight hours a day and do not take any protective measures in work place, and hence the occupational exposure to such genotoxic risk are high[4]. The present study aims to find the cytogenetic damage in exfoliated buccal cells obtained from petrol pump workers, using micronucleus (MN) assay.

The aim of our study was to determine the genotoxic effect of exposure to petroleum products on petrol pump workers using micronuclei assay and compare it with controls**.**

II. MATERIALS AND METHODS

Study period: Three months

Type of study: Observational study

Study Population: Petrol pump workers of Namakkal district

Sample size: 160

Type of sample: Buccal smear

METHODS:

The proposed study is to be conducted in the Department of Oral Pathology, Vivekanandha Dental College for Women, Tiruchengode.

SELECTION OF PATIENTS: Simple Random sampling

INCLUSION CRITERIA

- Petrol pump workers who has been working in petrol pump stations.

EXCLUSION CRITERIA

- Healthy subjects without the habit of alcohol and tobacco chewing and smoking habits.
- Subjects with systemic disease, Endocrine disease and known immunological diseases for the past one year.

Institutional Ethical Committee (IEC):

The study details has been presented in IEC and clearance certificate was obtained **(IEC no - VDCW/IEC/240/2021)**.

Sample Collection

The study sample includes 80 subjects in the age group of 21-60 years (Exposed group).The control group consisted of 80 individuals without clinically observed lesions and without any tobacco habits (Unexposed group I). The petrol pump workers who are exposed were further grouped as without the habits of smoking, chewing tobacco. (Exposed Group II, n=40) and those with habit of chewing tobacco and smoking in (exposed group III, n=40). These 160 subjects were selected from different petrol pumps in Namakkal district. Smoking habit and tobacco usage by the groups were evaluated using a prevalidated questionnaire.

Subjects were asked to rinse their mouth with water before sampling. The oral smear was taken from the buccal mucosa using a cytobrush .It was then transferred into centrifuge tubes with Phosphate Buffered Saline (PBS) at pH 7.2 and centrifuged for 10 minutes at 1500 rotations per minute (rpm). Supernatant was replaced with 5 ml of fresh PBS solution, centrifuged for 10 minutes at 1500 rpm and repeated twice. This supernatant

is discarded and the pellet smeared onto clean microscopic slides, air dried for10 minutes and fixed in ethanol and acetic acid in the ratio of 3:1 for 10 minutes. Slides was then stained using RAPID PAP for bright field microscopic analysis and evaluated using classification for nuclear abnormalities by Tolbert et al., [4] to determine the micronucleus (MN) frequencies. The results were tabulated and subjected to statistical analysis.

Criteria for the inclusion of cell for counting

1. Cytoplasm intact and lying relatively flat
2. Little or no overlap with adjacent cells and no debris.
3. Nucleus normal and intact, nuclear perimeter smooth and distinct.

Tolbert *et al* criteria parameters for identifying micronucleus are as follows [5].

1. Round smooth perimeter suggestive of a membrane.
2. Less than a third the diameter of associated nucleus, but large enough to discern shape and colour.
3. Staining intensity similar to nucleus.
4. Texture similar to nucleus
5. Same focal plane as nucleus and absence of overlap with or bridge to nucleus.

STATISTICAL ANALYSIS

The statistical analysis was done using SPSS VERSION 25.0. The Mann whitney U test was used to compare the mean MNC between the control and exposed group. The Willcoxon Signed rank test was used to compare the mean MNC between the group II – A and group II – B. The Krukal wallis test was used to compare between the mean MNC with the experience of control group and Mann Whitney U test was used to compare between the mean MNC with the experience of group II, group II – A, group II – B respectively. The statistical significance was kept at p – value less than 0.05.

III. Observations and Results

A total number of 100 cells were counted from each slide for the determination of micronuclei from control group and exposed group respectively. When stressed cells divide, chromosomal fragments lag behind and are excluded from the main nuclei in the daughter cells. These fragments form their own membranes and appear as feulgen-specific bodies termed as micronucleus in the cell cytoplasm.

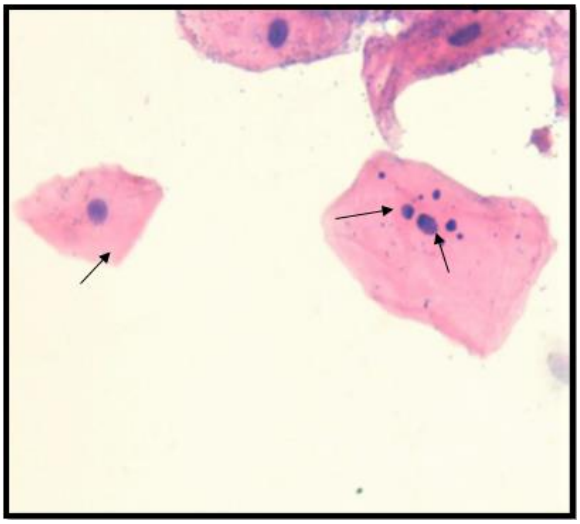

Figure 1 - Image shows exfoliated cell with micronuclei. (Magnification – 40X)

Table 1: Gender and age distribution of the subjects

Control (Group I)			**Exposed (Group II)**		
Gender	N(%)		Gender	N(%)	
Total	80		Total	80	
Men	52(65)		Men	65(81.2)	
Women	28(35)		Women	15(18.8)	
Age		**Mean ±SD**			**Mean± SD**
Total	80	35.4375±11.48	Total	80	31.9 ±10.11
18 -28 years	27(33.8)	24.03 ±2.91	**18 -28 years**	38(47.5)	23.3 ±3.56
29 - 38 years	26(32.5)	33.46±3.29	**29 - 38 years**	17(21.2)	32.3 ±2.6
39 - 48 years	15(18.8)	42.6±2.31	**39 - 48 years**	17(21.2)	41.9 ±2.46
49 - 58 years	7 (8.8)	54.14±2.74	**49 - 58 years**	8(10)	51.12 ±1.61
59 - 68 years	5 (6.2)	60.2± 1.93	**59 - 68 years**		

Cytological observation in study group

Table 2: Comparison of the MNC between the (control) Group I and (exposed) Group II

MNC	**N**	**Mean±SD**	**p value**
Group I	80	4.81±2.38	**0.000***
Group II	80	21.48±10.3	

*Significant at $p < 0.05$

Table 3: Comparison of the MNC between the exposed group

MNC	N	Mean±SD	p value
Group II - A	40	13.32±3.62	0.000*****
Group II - B	40	29.62±8.09	

*Significant at $p < 0.05$

Table 4 Comparison of the mean MNC with the exposure of the group –II subjects

Experience	N	Mean±S D	p-value
Less than 10 years	67	18.57±7.84	0.000*
More than 10 years	13	36.46±8.36	

*Significant at $p<0.05$

Table 5 Comparison of the mean MNC with the exposure of the group II-A and group II-B

Experience	Group II A N	Mean±S D	p-value
Less than 10 years	30	12.93±3.43	0.233
More than 10 years	10	14.50±4.09	
Experience	**Group II B N**	**Mean±S D**	**p-value**
Less than 10 years	27	26.33±5.60	**0.000***
More than 10 years	13	36.46±8.36	

*Significant at $p <0.05$

In the present study inter comparison of mean values for micronuclei using PAP stain between exposed petrol pump workers with habits of smoking and tobacco chewing ,exposed group without habits and control groups were done Demographic characteristics of the study subjects are shown in Table I. Age and sex ratio were nearly similar in both groups.

Micronuclei frequency of the study groups are given in Table II and Table III. Assessment of micronuclei frequencies in exfoliated buccal cells have shown that MN count was significantly higher($p<0.05$) in exposed group (21.48±10.3) compared to the unexposed group(4.81±2.38) .Among the exposed population a significantly high($p < 0.05$) MN frequency was observed in the exposed workers with habit of smoking and tobacco chewing (29.62±8.095) when compared to the exposed workers without these habits (13.32±3.619).

The frequency of nuclear abnormalities in women was found to be non-significant compared to men in our study as the population of male workers were 65% compared to 35% in the control group. In the exposed population the male population was 32% and 33% in group A and group B compared to 8% and 7% respectively.

The effect of exposure to petroleum fumes on micronuclei detection

In the present study, the mean MNC of the exposed subjects with exposure for more than 10 years to petroleum products (36.46±8.36) was significantly higher ($p < 0.05$) when compared to those workers with less than 10 years of exposure (18.57±7.84). A comparison of micronuclei count between exposed population with habits of smoking and tobacco chewing have shown a statistically significant high frequency than the exposed group without these habits. Also the frequency was found to be significantly higher (at $p <005$)in pump workers with longer duration of exposure in group B(26.33±5.60) when compared to workers with duration of exposure less than 10 years in Group B (36.46±8.36).

IV. Discussion

Petrol pump workers are persistently exposed to petroleum petrochemical products and its derivatives mainly through nasal or oral inhalation of the volatile fraction during vehicle refuelling, inhalation of automobile exhaust for long period of time as well as life style habits like smoking and tobacco chewing [6].

In India, the petrol pumps workers refill the vehicles uninterruptedly on a daily basis, with long working hours and hence are at high risk of having adverse health effects due to inhalation of volatile products like benzene, toluene, ethyl-benzene, xylene, *etc*. Petrol station workers are also liable to absorb the products emitted by engines. Most of the inhaled petroleum metabolites are getting absorbed in the body and further lead to many health hazards such as cancer, neurological diseases and teratogenic effects [7]. It is well established that one of the primary target tissue to get exposed to the inhaled gases is the buccal cavity and previous studies have established, buccal cavity study to be convenient for large-scale

monitoring studies in occupational and environmental toxic exposures[8].

The exfoliated buccal mucosa cells are potential sources for bio monitoring the exposure to the occupational and environmental hazards due to the direct route of exposure to pollutants in human population. The cells could be easily collected from the mouth by a non-invasive procedure. Also these buccal cells are potent enough to metabolize the carcinogen compounds to reactive substances .Since, the cells express the genotoxic effects, they are used to reveal the occupational exposure and compounds effects on MN formation.

The buccal mucosal cytology has a set of biomarkers that focuses on the number of micronucleus as well as the types of cells based on the nuclear morphology and cytological features that designate the stages of DNA damage and cell death in exfoliated buccal cells [9].

Epidemiological studies also showed a clear relationship between the increase in micronuclei frequency and exposure to benzene and its metabolites [10, 11]

The mean MN count in exposed group was 21.48±10.3 compared to the unexposed group 4.81±2.38 with p <005 .Celik *et al* have also demonstrated increased MN frequencies in exfoliated buccal cells of exposed petrol pump workers 1.34± 0.80 when compared to control subjects 0.47 ±003 [12].Studies of Bukvic *et al* have showed significant micronuclei count difference in peripheral lymphocytes of petrol station workers and Santos-Mello observed a significant increase in chromosome deletion [13].

However Surralles *et al* found no significant difference in micronuclei frequency in the buccal cells of exposed workers and controls. Similar results were observed in studies of Carere *et al* and Pitarque *et al* among petrol station workers [14, 15]. Hadnagy studied the cytotoxic and genotoxic properties and effects of different components of automobile emission.[16] Sarto *et al* and Piyathilake *et al* has reported the increase in MN frequency in buccal cells due to smoking [17] .In our study the exposed population with habits of smoking and tobacco chewing 29.62± 8.09 have shown a significant induction of MN compared to exposed group without habits 13.32±3.62.This could be due to the effects of constituents of petroleum fumes, fumes from vehicle exhaust and other petroleum products of various sources and smoke from cigarettes. The cigarette and smokeless tobacco also proved its contemporary effect and caused nuclear degeneration and has impressed the count of micronuclei [18].

The study revealed that the micronuclei count was significantly higher in those exposed workers with duration of exposure more than 10 years, than in those exposed for less than 10 years. This could be attributed to the degree of genotoxic effect due to longer period of exposure to the fumes of petroleum products. Increased micronuclei frequency in exfoliated buccal cells of petrol pump workers may be due to the genotoxin benzene and its metabolites present in automobile exhaust and tobacco smoke. The inhaled benzene enters the blood stream through the lungs, from where about 70% of it gets exhaled while remaining gets detoxified in the liver and converts into a number of metabolite like benzene-oxide, phenol and catechol which are toxic to the body [19] .

Anderson *et al*. demonstrated that benzene and its metabolites induce DNA damage in human lymphocytes. Also chromosomal aberrations in circulating lymphocytes are considered to be risk factor for cancer [20]. Liou *et al*. demonstrated that people having chromosome type aberrations in their lymphocytes were at higher risk of having cancer.

Brandt *et al*. and Zhou *et al*. have demonstrated genotoxic effect in workers exposed to low levels of benzene from petrol [21]. Various studies have concluded that benzene is a multi-potential carcinogen capable to inducing tumours and low level of benzene affects human immuno-defence.

Another factor that could affect MN frequency in petrol pump workers with chronic exposure to petrol fumes has been found to be the gender of the exposed group. Because of the difference in male and female physiology, the degree of micronuclei count may differ [22]. In our study no significant difference in the frequency of nuclear abnormallities was observed in men compared to women. A study on Japanese population also observed a similar result where as a meta analysis study on DNA damage reported a higher MN frequency among male compared to female workers [23,24]. The reason for non significance among male and female exposed workers may be because the number of female subjects in our study is less than male subjects. Also the duration of service and work schedule in the petrol station for female

workers will be less due to socioeconomic reasons in India.

V.Conclusion

Micronuclei (MN) assay has a definite advantage in terms of simplicity, less ease technique sensitive, economical and minimum time consuming procedure. MN assay can be used as a simple chair side or mass screening method to assess and identify patients with early genomic damage. The present study ascertains the role of micronuclei assay and oral exfoliative cytology in bio monitoring the genotoxic changes in oral epithelial cells in subjects with occupational exposure to petroleum vapours in petrol pump attendants. Application of DNA specific stains can increase the sensitivity of the assay in detecting micronuclei in exfoliated buccal cells.

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