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Biomonitoring genetic instability in normal healthy population using a simple cytogenetic marker – micronucleus test

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Abstract: Micronuclei in epithelial cells are widely used as biomarkers for monitoring genetic instability in humans. In this study, we have evaluated the buccal cells of 144 normal healthy individuals. The frequency of micronuclei was analysed and their relationship to different confounding factors (age, gender, diet and life style habits) was determined. The results show a significant increase in the observed frequency of micronuclei with respect to age. Males show a comparatively high percentage of micronuclei. Non-vegetarian diet, smoking and alcohol consumption are also suggestively significant.

Keywords: Buccal cell micronuclei; Age; Gender; Diet; Smoking; Alcohol consumption

INTRODUCTION

Micronucleus is the small nucleus that forms whenever a chromosome or a fragment of a chromosome is not incorporated into one of the daughter cell during cell division. These were first described by Howell and Jolly in late 1800's and early 1900's as Feulgen-positive nuclear bodies in human reticulocytes, representing chromosomes separated from the mitotic spindle. They attributed the formation of micronuclei to the presence of accentric fragments and the exclusion of these fragments from daughter nuclei at telophase **(1)**.

In early 1970's, Boller and Schmidt suggested the term 'Micronucleus'. The presence of micronuclei in cells has been associated with chromosomal instability. Micronuclei can be a manifestation of an identifiable event which could be associated with various genetic and environmental factors **(2)**. Micronuclei is known to provide a measure of both chromosome breakage and chromosome loss, and it has been shown to be at least as sensitive an indicator of chromosome damage as classical metaphase chromosome analysis.

Micronucleus Assay was thus identified to evaluate the presence and formation of micronuclei in various cell types. This assay has been used in many epidemiological studies as an effective indicator of chromosomal damage in epithelial cells and lymphocytes. The buccal cell micronuclei assay was first proposed by Stich et al. **(3, 4)** as a useful biomarker of genetic damage caused by lifestyle habits, environmental exposure, medical procedures and inherited genetic defects.

Humans are constantly exposed to reactive chemicals and agents derived from exogenous sources such as food, air pollution, tobacco smoke or ionizing radiation, and also from endogenous sources including reactive oxygen species (ROS) formed from mitochondrial respiration, cellular metabolism and the immune defense system. By reaction with cellular biomolecules (DNA) genetic alterations such as chromosomal aberration and formation of micronuclei in cell cytoplasm, are the early biological effects which may lead to an increased cancer risk or aging **(5)**. The body has effective defense and repair systems for genetic material protection, maintenance and repair **(6)**. However, these mechanisms are not infallible. Genetic damage occurs, may escape repair, and this may lead to mutation, with disturbed cellular metabolism or cell death. What exactly contributes to this damage if there is, in normal population is a question that needs to be answered.

None the less, there are plausible contributors that effect DNA which generally goes ignored. There are studies linking DNA damage to ageing, lifestyle especially the diet, occupation, family history (susceptibility to particular disease) and environmental agents **(7, 8)**. There are several factors that could be associated with intra and inter-individual variations in genetic instability. The exact contributing factor needs to be established.

For example aging in humans appeared to be associated with genetic instability **(9)**, which may be due to adverse endo-and exogenous conditions which result in increased cytogenetic level- is reflected by increased frequency of chromosomal aberrations **(10)**. Moreover, there are studies which have reported that the pattern of this age-related increase in the genetic instability differed between men and women.

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MATERIALS AND METHODS

A total of 172 individuals from normal healthy Indian population were selected for the study. All subjects participated voluntarily; they were informed of the objectives of the study to which they gave their consent. To avoid possible bias and to assure subject confidentiality, the samples were coded. All the participants completed a questionnaire (**table I, II**) and provided the buccal sample. The questionnaire elicited demographic data (age and gender), food habits, medical status (including history of health problems), drinking, exercising and smoking habits, and prior or current exposure to medication or environmental agents that could affect the micronuclei assay (e.g., X-rays, anti-cancer chemotherapy, etc).

Table – I: Questionnaire for males

NAME: _____

DATE OF BIRTH: _____ AGE: _____ RELIGION: _____

OCCUPATION: _____

NO. OF YEARS OF SERVICE: _____

NO. OF WORKING HOURS/DAY: _____

MARITAL STATUS:

MARRIED/ SINGLE	NO. OF YEARS OF MARRIAGE	NO. OF CHILDREN	AGE OF CHILDREN

VEGETARIAN/ NON VEGETARIAN: _____

FREQUENCY OF CONSUMPTION (IF NON VEGETARIAN): _____

PHYSICAL ACTIVITIES:

PHYSICAL EXERCISES	SPORTS	YOGA	MEDITATION	ANY OTHER

ALCOHOLIC/ NON ALCOHOLIC: _____

FREQUENCY OF ALCOHOL CONSUMPTION: _____

SMOKER/ NON SMOKER: _____ FREQUENCY OF SMOKING: _____

MEDICAL HISTORY: _____

LAST MEAL TIME AND NATURE: _____

ARE YOUR PARENTS CONSANGINEOUSLY RELATED? _____

TIME OF SAMPLE COLLECTION: _____ DATE: _____

CONTACT NO.: _____ SIGNATURE: _____

Table – II: Questionnaire for females

NAME: _____

DATE OF BIRTH: _____ AGE: _____ RELIGION: _____

OCCUPATION: _____

NO. OF YEARS OF SERVICE: _____

NO. OF WORKING HOURS/DAY: _____

MARITAL STATUS:

MARRIED/ SINGLE	NO. OF YEARS OF MARRIAGE	NO. OF CHILDREN	AGE OF CHILDREN

VEGETARIAN/ NON VEGETARIAN: _____

FREQUENCY OF CONSUMPTION (IF NON VEGETARIAN): _____

PHYSICAL ACTIVITIES:

PHYSICAL EXERCISES	SPORTS	YOGA	MEDITATION	ANY OTHER

ALCOHOLIC/ NON ALCOHOLIC: _____

FREQUENCY OF ALCOHOL CONSUMPTION: _____

SMOKER/ NON SMOKER: _____ FREQUENCY OF SMOKING: _____

DATE OF LAST MENSTRUAL PHASE: _____ REGULAR/ IRREGULAR

MEDICAL HISTORY: _____

LAST MEAL TIME AND NATURE: _____

ARE YOUR PARENTS CONSANGINEOUSLY RELATED? _____

TIME OF SAMPLE COLLECTION: _____ DATE: _____

CONTACT NO.: _____ SIGNATURE: _____

Table A: Sample collection for scoring of micronuclei from human subjects (coded) in the age group (16 – 26) years

SL.NO	CODE	AGE	GENDER	FOOD AND LIFE STYLE HABITS		
				FOOD (V/NV)	SMOKING (S/NS)	ALCOHOL (A/NA)
1	MN 04	20	M	NV	NS	NA
2	MN 05	19	M	NV	NS	A
3	MN 16	26	F	NV	NS	NA
4	MN 19	21	F	NV	NS	NA
5	MN 20	20	M	NV	NS	NA
6	MN 21	23	F	NV	NS	NA
7	MN 24	19	F	V	NS	NA
8	MN 26	20	M	NV	NS	A
9	MN 27	19	M	V	NS	NA
10	MN 28	20	F	NV	NS	NA
11	MN 30	22	M	NV	NS	NA
12	MN 31	20	M	NV	NS	NA
13	MN 32	19	M	V	NS	NA
14	MN 34	21	F	NV	NS	NA
15	MN 35	21	M	NV	NS	NA
16	MN 36	20	M	NV	S	A
17	MN 37	21	F	V	NS	NA
18	MN 38	21	F	NV	NS	NA
19	MN 41	20	M	NV	NS	A
20	MN 42	20	F	NV	NS	NA
21	MN 43	21	F	NV	NS	NA

22	MN 48	23	F	NV	NS	NA
23	MN 55	23	F	V	NS	NA
24	MN 56	24	F	V	NS	NA
25	MN 57	23	F	V	NS	NA
26	MN 58	26	F	NV	NS	NA
27	MN 66	23	F	V	NS	NA
28	MN 68	24	M	NV	NS	NA
29	MN 70	26	F	V	NS	NA
30	MN74	26	F	V	NS	NA
31	MN 144	20	M	V	NS	NA
32	MN 145	20	M	NV	NS	NA
33	MN 146	20	M	V	S	NA
34	MN 147	20	M	V	NS	NA
35	MN 148	20	M	V	NS	NA
36	MN 149	21	M	NV	S	NA

Table B: Sample collection for scoring of micronuclei from human subjects (coded) in the age group (27 – 36) years

SL.NO	CODE	AGE	GENDER	FOOD AND LIFE STYLE HABITS		
				FOOD (V/NV)	SMOKING (S/NS)	ALCOHOL (A/NA)
1	MN 02	29	F	NV	NS	NA
2	MN 03	34	M	NV	NS	NA
3	MN 06	32	F	V	NS	NA
4	MN 07	32	F	V	NS	NA
5	MN 08	27	F	V	NS	NA
6	MN 13	28	F	NV	NS	NA
7	MN 14	28	F	V	NS	NA
8	MN 15	35	M	NV	NS	A
9	MN 21	31	M	NV	NS	NA
10	MN 23	29	F	NV	NS	NA
11	MN 25	30	M	NV	NS	NA
12	MN 33	32	F	NV	NS	NA
13	MN 44	33	M	NV	NS	A
14	MN 45	27	M	V	NS	NA
15	MN 47	32	M	V	NS	NA
16	MN 49	30	F	NV	NS	NA
17	MN 59	32	F	V	NS	NA
18	MN 60	32	M	NV	NS	NA
19	MN 63	33	F	NV	NS	NA
20	MN 64	35	F	NV	NS	NA
21	MN 65	31	F	NV	NS	NA
22	MN 69	30	M	NV	NS	NA
23	MN 72	35	F	NV	NS	NA
24	MN 73	32	M	NV	NS	NA
25	MN 75	34	M	V	NS	NA
26	MN 76	32	M	NV	NS	NA
27	MN 77	34	F	V	NS	NA
28	MN 78	31	M	NV	NS	NA
29	MN 81	32	F	V	NS	NA
30	MN 83	32	F	NV	NS	NA
31	MN 84	32	M	NV	S	A
32	MN 88	33	F	V	NS	NA
33	MN 92	28	M	NV	NS	NA
34	MN 94	32	M	NV	NS	NA
35	MN 100	30	M	NV	NS	NA
36	MN 101	34	M	V	NS	NA

Table C: Sample collection for scoring of micronuclei from human subjects (coded) in the age group (37 – 46) years

SL.NO	CODE	AGE	GENDER	FOOD AND LIFE STYLE HABITS		
				FOOD (V/NV)	SMOKING (S/NS)	ALCOHOL (A/NA)
1	MN 01	38	F	V	NS	NA
2	MN 09	37	F	NV	NS	NA
3	MN 12	42	F	V	NS	NA
4	MN 18	38	M	NV	NS	NA
5	MN 22	36	M	V	NS	NA
6	MN 46	39	M	V	NS	NA

7	MN 50	40	F	NV	NS	NA
8	MN 51	37	F	NV	NS	NA
9	MN 52	37	M	NV	NS	NA
10	MN 53	38	F	V	NS	NA
11	MN 61	37	F	NV	NS	NA
12	MN 67	37	F	V	NS	NA
13	MN 79	41	M	NV	S	NA
14	MN 80	36	F	V	NS	NA
15	MN 85	41	M	NV	NS	NA
16	MN 87	38	M	V	NS	NA
17	MN 89	37	M	V	NS	NA
18	MN 90	43	M	NV	NS	NA
19	MN 96	38	M	V	NS	NA
20	MN 97	43	M	V	NS	NA
21	MN 98	45	F	V	NS	NA
22	MN 108	37	F	NV	NS	NA
23	MN 109	40	F	V	NS	NA
24	MN 110	44	F	V	NS	NA
25	MN 111	43	M	NV	NS	NA
26	MN 112	36	F	V	NS	NA
27	MN 113	39	F	V	NS	NA
28	MN 114	39	M	V	NS	NA
29	MN 115	43	M	V	NS	NA
30	MN 124	36	F	V	NS	NA
31	MN 129	38	M	V	NS	NA
32	MN 131	37	M	V	NS	NA
33	MN 168	42	F	NV	NS	NA
34	MN 153	43	F	V	NS	NA
35	MN 136	45	M	V	NS	NA
36	MN 165	42	M	NV	NS	A

Table D: Sample collection for scoring of micronuclei from human subjects (coded) in the age group above 47 years

SL.NO	CODE	AGE	GENDER	FOOD AND LIFE STYLE HABITS		
				FOOD (V/NV)	SMOKING (S/NS)	ALCOHOL (A/NA)
1	MN 169	60	M	V	NS	NA
2	MN 86	53	F	V	NS	NA
3	MN 93	53	F	V	NS	NA
4	MN 99	68	M	V	NS	NA
5	MN 104	58	M	V	NS	NA
6	MN 105	53	F	V	NS	NA
7	MN 106	57	F	V	NS	NA
8	MN 107	65	M	V	S	NA
9	MN 120	55	F	NV	NS	NA
10	MN 116	48	M	V	NS	NA
11	MN 118	54	M	V	NS	NA
12	MN 119	53	F	V	NS	NA
13	MN 121	63	F	NV	NS	NA
14	MN 123	48	M	NV	NS	A
15	MN 125	51	M	NV	S	NA
16	MN 128	60	F	V	NS	NA
17	MN 130	60	M	NV	NS	NA
18	MN 132	73	M	V	NS	NA
19	MN 133	60	F	V	NS	NA
20	MN 135	50	M	NV	NS	NA
21	MN 137	50	M	V	NS	NA
22	MN 138	53	F	V	NS	NA
23	MN 140	58	F	V	NS	NA
24	MN 139	51	F	V	NS	NA
25	MN 141	54	F	V	NS	NA
26	MN 164	57	M	NV	NS	NA
27	MN 163	54	F	NV	NS	NA
28	MN 160	62	F	NV	NS	NA
29	MN 166	70	M	V	NS	NA
30	MN 167	50	M	NV	S	A
31	MN 162	48	M	NV	NS	A
32	MN 156	61	F	NV	NS	NA
33	MN 159	55	F	NV	NS	NA
34	MN 155	60	M	V	NS	NA
35	MN 154	57	F	V	NS	NA
36	MN 151	51	M	V	NS	NA

Table 1: Final percentage frequency distribution of micronuclei evaluated

Age Group	Human subjects (code)	Gender	Food and life style habits			No. of cells showing MN	Avg. No. of MN	% of MN
			Smoking	Diet	Alcohol consumption			
I	MN05	M	NS	NV	A	6	5	0.6
II	MN33	F	NS	NV	NA	2	2	0.2
III	MN not observed							
IV	MN118	M	NS	V	NA	1	1	0.1
	MN125	M	S	NV	NA	1	1	0.1

Group I = 16 - 26 yrs
Group II = 27 - 36 yrs
Group III = 37 - 46 yrs (MN not observed)
Group IV = above 47 yrs
MN = Micronuclei
Avg. frequency of MN = total no. of MN/ total no. of cells showing MN
% of MN = no. of cells showing MN/total cells scored x100

NV = non vegetarian NA= non alcoholic NS= non smoker
V = vegetarian A= alcoholic S= smoker

Table 2: Positive incidence of micronuclei in healthy human population studied.

Variable	Total no. of subjects	Number of subjects showing the presence of micronuclei	Incidence %	P value
Age in years				
• 16-26	36	1	2.77	1.000
• 27-36	36	1	2.77	1.000
• 37-46	36	0	0.0	0.311
• >47	36	2	5.56	0.307
Gender				
• Male	72	3	4.17	0.467
• Female	72	1	1.38	0.473
Diet				
• Vegetarian	72	1	1.38	0.473
• Non-Vegetarian	72	3	4.17	0.467
Smoking				
• Yes	8	1	12.50	0.093+
• No	136	3	2.21	0.691
Alcohol				
• Yes	11	1	9.09	0.201
• No	133	3	2.23	0.704
Total	144	4	2.77	-

(Positive incidence of Micronuclei is associated with higher Age group, Male population, Non-vegetarian diet, Smoking and Alcohol consumption.)
+ Suggestive significance (P value: $0.05<P<0.10$)
* Moderately significant (P value: $0.01<P \leq 0.05$)
** Strongly significant (P value: $P \leq 0.01$)

It is necessary to emphasize that due to extrinsic factors, some data were missing, and thus the final number of individuals analyzed was lower, 144(**table A, B, C, D**).

The population was categorized into four groups based on age [intervals: group I (16-26), group II (27-36), group III (37-46), group IV (>47 years)] and equal number of subjects were considered in each group. Care was taken to have equal distribution of gender in each age group for comparative statistical analysis. Distribution of subjects with respect to the confounding factors (food and life style habits) was also taken into account to study its effect on micronuclei assay.

Immediately before the sample collection, the individuals were asked to rinse their mouth with drinking water. Buccal epithelial cells were obtained from the oral cavity by gently scraping the mucosa inside each cheek separately with wooden spatulas. The cells were transferred into 10ml centrifuge tubes containing 3ml buffer (0.1M/L EDTA, 0.01M/L Tris and 0.02M/L NaCl). The cells were washed thrice in the buffer solution by centrifugation at 10000 RPM. Now the supernatant decanted and the cell pellet was transferred to a clean microscopic glass slide using a micropipette/Pasteur's pipette. A smear was prepared and the slide air dried. The cell sample was fixed in Carnoy's fixative [3:1 (methanol: acetic acid)] for 2-3 minutes and the fixative was drained later. The slides were stained with Geimsa and left overnight. These slides were then washed with distilled water neatly and allowed to air dry again just before screening. Microscopic analysis was performed on EN-make Binocular Microscope at a magnification of 400X. Micronuclei were determined according to criteria established by Stich and Rosin **(11, 12)**.

Further, the examination and scoring of ~ 1000 cells per sample is labor intensive; it would not be unexpected to find that the counting procedure is prone to error. The frequency of micronuclei in buccal cells of healthy subjects is low (<0.5%); thus, a few misread false- positive cells would be statistically significant.

To avoid differences during scoring, microscopic analyses was carried out by the same team.

RESULTS AND OBSERVATIONS

The following are the results based on different parameters taken into account to analyze the data (**Figure 1**).

The number of micro-nucleated cells and the percentage of micronuclei obtained among the different categories are represented in **Table 1** (**Illustration 1, 2, 3, 4**).

Regarding age the average difference among the four groups does not represent biological significance. However there is a slight increase in the observed frequency of micronuclei in group IV (>47 years) (**Table 2**).

With respect to gender, the observations show that the percentage of micronuclei in males is more than in females (**Table 2**).

In relation to food habits, the results indicate that diet has a direct influence on micronuclei frequency. Nevertheless, life style also has an impact. Smoking is suggestively significant (P=0.093) with a 12.5% incidence out of the total number of smokers (**Table 2**).

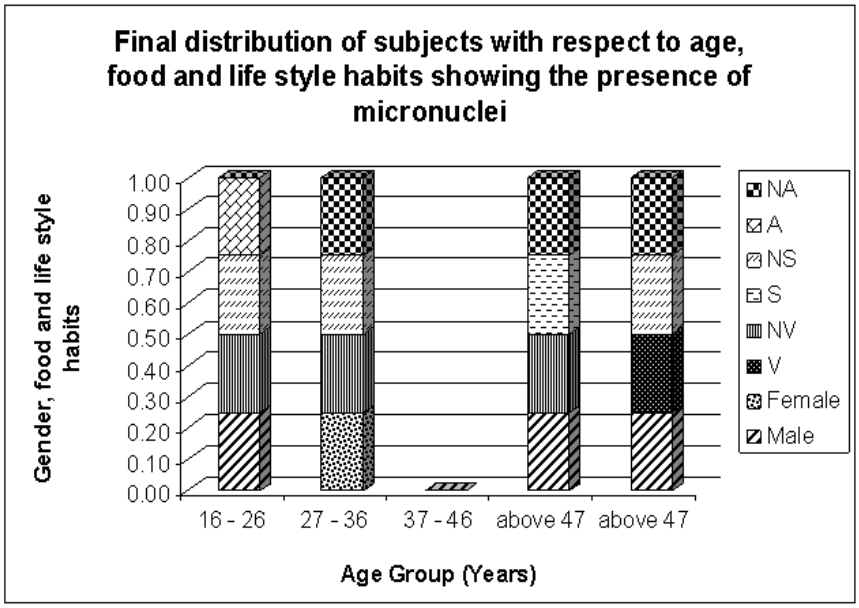

Figure 1: Final distribution of subjects with respect to age, food and life style habits showing the presence of micronuclei. (Refer Table: 1)

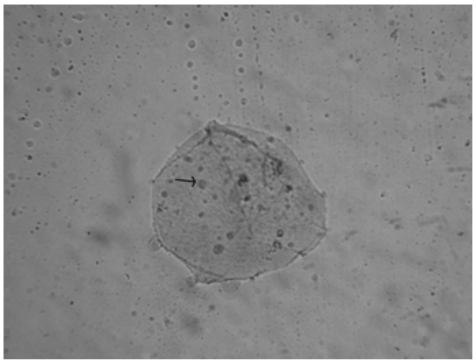

Illustration 1: Buccal epithelial cell showing micronuclei- sample code MN 05

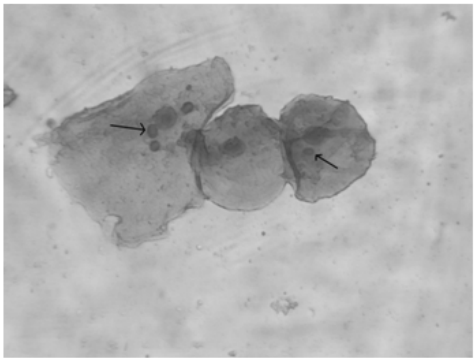

Illustration 2: Buccal epithelial cell showing micronuclei- sample code MN 33

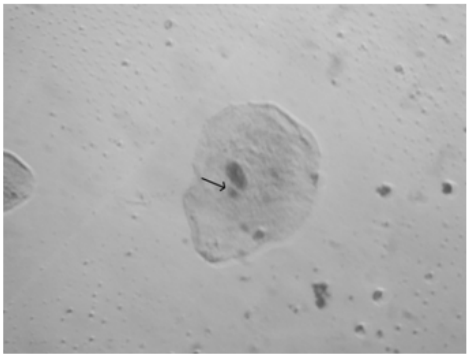

Illustration 3: Buccal epithelial cell showing micronuclei- sample code MN 118

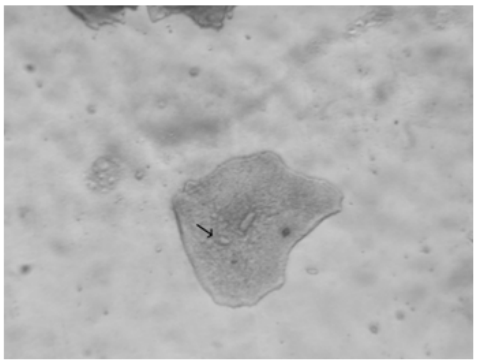

Illustration 4: Buccal epithelial cell showing micronuclei- sample code MN 125

DISCUSSION AND CONCLUSION

In summary, the results of this study indicate that the four normal healthy population groups do not reveal a significant cytogenetic damage, as measured by the micronuclei assay in the buccal epithelial cells. Classification of subjects with respect to age group was done for comparative statistical analysis and also to find out the tendency of formation of micronuclei with respect to various confounding factors. A high degree of heterogeneity was observed between the four populations studied for the frequency of micronuclei. This is in agreement with the results obtained (**Figure 1**).

As previously reported **(13, 14, 15)** age is strongly associated in a positive way with micronuclei frequency. In our overall data with respect to number of individuals showing the presence of micronuclei (**Table 2**), there is a slight increase in the group IV (age interval: >47). Whereas group I, II and III did not show remarkable differences though nothing was observed in group III.

Many authors have reported an age related increase in micronuclei frequency in both the sexes **(16, 17)**. Our analysis shows that the incidence of micronuclei in males is observed to be 4.17 [MN 05 – parents consangeniously married; MN118 – hypertensive and highly obese; MN 125 – NV and Smoker] and in females 1.38 [MN 33 – NV and Parents Hyperglycemic] (**Table 2**).

Diet in correlation with age was found to be significantly associated with micronuclei frequency. Higher age group is associated with Micronuclei formation; however higher age associated with non vegetarian diet has a greater risk.

Smoking is reported to increase the micronuclei frequency in buccal cells **(18, 19, 20)**. From our data, it is clear that group I (8.3%) and group IV (8.3%) had the highest percentage of smokers. Alcohol consumption was observed to be higher in the younger age group (group I) and not statistically significant in the other groups. Thus, although smoking and alcohol consumption may be a possible factor to explain the high frequency of micronuclei, in our study statistical analysis indicates a suggestive significance for smoking (**Table 2**) and not statistically very significant for alcohol consumption. The study shows that even in a normal healthy population, there is a positive incidence of Micronuclei when correlated with higher Age group, Gender (male), Non-vegetarian diet, Smoking and Alcohol consumption.

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