IOSR Journal of Dental and Medical Sciences (IOSR-JDMS)
e-ISSN: 2279-0853, p-ISSN: 2279-0861.Volume 19, Issue 4 Ser.6 (April. 2020), PP 56-61
www.iosrjournals.org

Evaluation of Micronuclei in Occupational Exposure to Formaldehyde: A Study in Subjects In Pathology Laboratories

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ABSTRACT:
BACKGROUND OF THE STUDY:
Formaldehyde is the one commonly used in histopathology department. Since 2006, International Agency for Research on Cancer (IARC) classifies formaldehyde as carcinogenic to humans, based on sufficient evidence in humans and in experimental animals. Invitro studies clearly indicated that FA can induce genotoxic effects in proliferating cultured mammalian cells. Some invitro studies detected changes in epithelial cells (nasal and oral) and in peripheral lymphocytes related to FA exposure.The pathologist and technician who undertake the grossing have an exposure to formaldehyde. Hence the present study was undertaken. Micronuclei frequency is being used as a marker for genotoxicity.
***OBJECTIVES**:*
To assess the genotoxic effect of occupational exposure to formaldehyde in subjects in pathology laboratory and to evaluate the micronuclei frequency in buccal mucosal cells of histopathology technicians and pathologists in the histopathology department
MATERIALS AND METHODS:
The exfoliated cells (buccal mucosa cells) are collected from each subject using wooden spatula. Exfoliated cells smeared onto the slides and sprayfixed (BioFix spray) and stained with Acridine Orange stain.
RESULTS:
The mean number of micronucleus of buccal mucosa cells in group exposed to formaldehyde and group not exposed was 7.13 ± 0.98 and 0.2 ± 0.088 respectively. The difference was statistically significant (p = 0.000). The percentage of the cells with micronucleus in formaldehyde exposed with history of exposure greater than 25 years (group 1), 10-25 years (group 2), less than 10 years (group 3), no exposure(group 4) was 14.38±.999, 5.93±.774, 2 ±.655, .2±.088 respectively. The difference between the MN count between group 1, 2 and 3 was statistically significant. Groups 3 and 4 didn't show statistically significant difference.
CONCLUSION:
The present study highlights that there is significant DNA damage in subjects exposed to FA. Appropriate protective measures have to be adopted.
KEYWORDS: Formaldehyde, micronuclei, acridine orange

Date of Submission: 02-04-2020 Date of Acceptance: 18-04-2020

I. Introduction

Formaldehyde (CH_2O) is the most simple and reactive of all aldehydes, is a colourless, reactive and readily polymerising gas at room temperature. Human studies have shown that chronic exposure to FA by inhalation is associated with eye, nose and throat irritation[1]. Invitro studies clearly indicated that FA can induce genotoxic effects in proliferating cultured mammalian cells[2]. Some invitro studies detected changes in epithelial cells and in peripheral lymphocytes related to FA exposure[3,4].

Frequency of micronucleus (MN) in buccal mucosal cells is being used to investigate local genotoxicity. MN is the most sensitive genetic end point for detection of FA induced genotoxicity. Thus, MN test with exfoliated cells could be a powerful tool for detection of local genotoxic effects in humans, which is fundamental for hazard identification and risk estimation[1].

Micronuclei are intracytoplasmic DNA staining bodies, caused due to double strand chromosomal aberrations. These damaged chromosomes in the form of acentric chromosome fragments are included in the daughter cells too as secondary nuclei, much smaller than the principal nucleus and are therefore called micronuclei. Among the various non invasive early detection tools, it is documented that micronuclei assay can be used as a diagnostic and prognostic marker which represent as as "internal dosimeter" to estimate exposure to genotoxic and carcinogenic agents[3,4].

Studies indicate that macroscopic examination of FA preserved anatomical specimens involve exposure to highest values. This occurs because precision and good visibility is required and as a consequence pathologists must lean over the specimen with consequent increase of proximity to FA emission sources[1].MN frequencies tend to rise with age because of progressive increase in spontaneous chromosome instability and the loss of efficiency in DNA repair mechanisms[2].

Since 2006, International Agency for Research on Cancer (IARC) classifies FA as carcinogenic to humans, based on sufficient evidence in humans and in experimental animals[3].

The aims and objectives of the present study was to assess the genotoxic effect of occupational exposure to formaldehyde in subjects in pathology laboratory and to evaluate the micronuclei frequency in buccal mucosal cells of histopathology technicians and pathologists in the histopathology department and to evaluate the time related effects of formaldehyde.

II. Materials and methods:

This quantitative, analytical study was conducted in multiple centers (different histopathology labs). Institutional ethical clearance was obtained prior to commencement of the study. The study group included 30 subjects which comprised of pathologists, lab technicians, lab assistants. The control group included 30 healthy volunteers. Those people who were willing to participate in the study, who are exposed to formaldehyde at work place for more than 5 years, between 30 – 60 years were selected. Participants with systemic illness ,under medications, with a history alcoholism, chewing habit and smoking were excluded.

The exfoliated cells (buccal mucosa cells) were collected from each subject using wooden spatula.Cells were smeared onto the slides and sprayfixed using Biofix spray and stained with Acridine Orange stain (Sigma Aldrich). 100µM Acridine Orange was added to the slides and was incubated in dark for 10 minutes,washed three times in PBS and viewed under Olympus CKX 41 microscope , CCD camera- Pro-5 (Trinocular research microscope). (Fig 1)

Hundred cells were scored from each individual. Only cells containing intact nuclei that are not clumped or overlapped were included in the analysis.

Data analysis:

The data was analyzed using t-test, ANOVA.

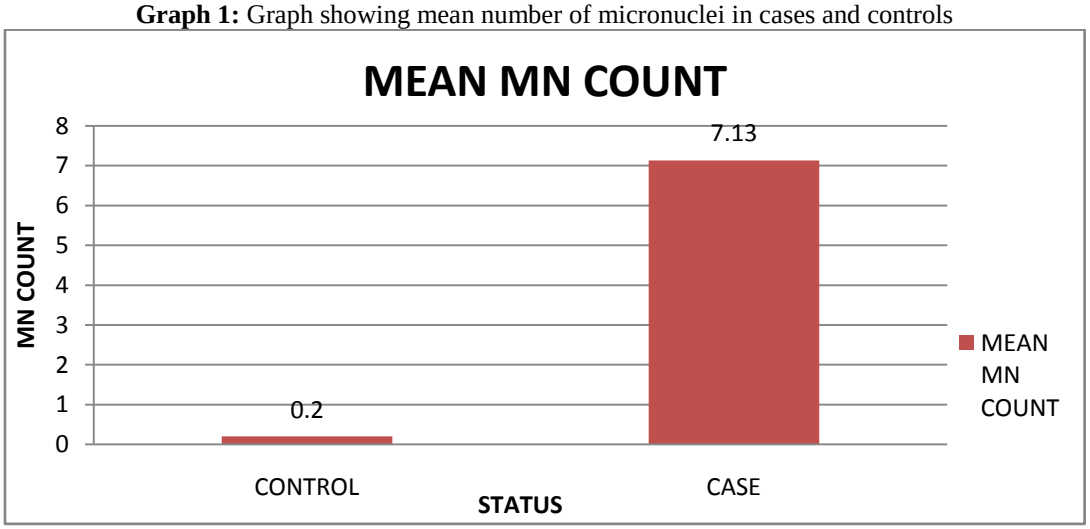
III. Results:

The mean number of micronucleus of buccal mucosa cells in group exposed to formaldehyde was 7.13 ± 0.98 and the group not exposed was 0.2 ± 0.088 respectively. The difference was statistically significant (p = 0.000). (Table 1, Graph 1)

Table 1: Distribution of micronuclei frequency in cases and controls:

	N	Mean MN Count	SD	Mean±SE
Control	30	0.2	0.484	0.2±.088
Case	30	7.13	5.38	7.13±.98

Graph 1: Graph showing mean number of micronuclei in cases and controls



The percentage of the cells with micronucleus in formaldehyde exposed individuals with history of exposure greater than 25 years (group 1), 10-25 years (group 2), less than 10 years (group 3), no exposure(group 4) was 14.38±.999, 5.93±.774, 2 ±.655, .2±.088 respectively. The difference between the MN count between group 1 and 2,3,4 was highly statistically significant with p = 0.000. The difference between the MN count between group 2 and 3 was statistically significant with p = 0.003. The difference between the MN count between group 3 and 4 was highly statistically significant with p = 0.000. Groups 3 and 4 didn't show statistically significant difference. (p= 0.029). (Table 2,3,4; Graph 2)

Table 2: Distribution of micronuclei count in duration of exposure

Duration of Exposure	**N**	**Mean MN Count**	**SD**	**Mean ± SE**
Greater than 25	8	14.38	2.825	14.38±.999
10-25 yrs	14	5.93	2.895	5.93±.774
Less than 10 years	8	2	1.852	2±.655
No exposure	30	0.2	0.484	.2±.088

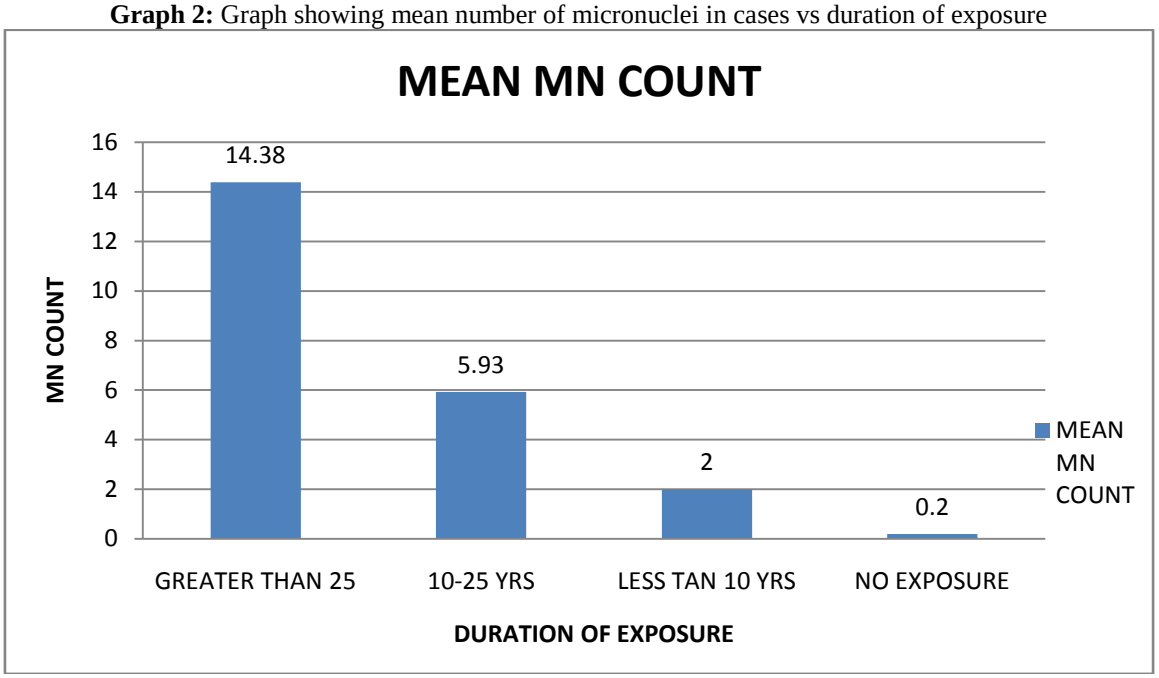
Table 3: Variation in micronuclei count between cases and controls were analyzed using t test and ANOVA

Group	Mean ± SE	P value
Cases and controls	6.933±0.986	0.0 highly significant)

Table 4: Variation in micronuclei count according to duration of exposure was also analyzed using t test

Groups	P value
Cases having more than 25 yrs experience And other groups(10-25 yrs, <10 yrs, controls)	0.000(highly significant)
Cases having 10-25 yrs experience and < 10 yrs experience	0.003(significant)
Cases having 10-25 yrs experience and controls	0.000(highly significant)
Cases having < 10 yrs experience and controls	0.029(not significant)

Graph 2: Graph showing mean number of micronuclei in cases vs duration of exposure



The mean number of micronucleus of buccal mucosa cells in pathologists having >25 years experience was 13.00 ± 1.15 and that of technicians was 15.20±1.39 respectively. The difference was not statistically significant (p =0.322).(Table 5)

Table 5: Distribution of micronuclei frequency in cases having >25 years experience

	profession	Mean± Std. Error	pvalue
MN Count	pathologists	13.00±1.15	0.322
	technicians	15.20±1.39	

IV. Discussion

The long-term exposures to formaldehyde, such as occupational exposures, are suspected to be associated with genotoxic effects. This can be evaluated by analysis of biomarkers[5,6,7]. Macroscopic examination of specimens is the task that involves higher exposure,because it requires a greater proximity to anatomical preparations impregnated with FA[3,8].

FA exposure and biomarkers of genotoxicity, namely micronuclei (MN) in lymphocytes and buccal cells, nucleoplasmic bridges and nuclear buds show a statistically significant association[2,3]. Exfoliated buccal cells are good source of tissue for monitoring human exposure to inhaled and ingested occupational and environmental genotoxicants[9]. Chromosome damage and effects on lymphocytes arise because formaldehyde escapes from sites of direct contact, such as the mouth, causing nuclear alterations in the lymphocytes of those exposed [10].

In humans, FA exposure is associated with an increase in the frequency of MN in buccal epithelium cells, as shown by the results presented here. Suruda et al., by his study on cytogenetic effects of formaldehyde exposure in students of mortuary science claim that although changes in oral and nasal epithelial cells and peripheral blood cells do not indicate a direct mechanism for carcinogenesis, they present evidence that DNA alteration took place. It can be concluded that FA is a cancer risk factor for those who are occupationally exposed in histopathology laboratories[11].

Studies in the buccal mucosa are preferred as, this is an easily accessible tissue for sampling cells in a minimally invasive manner and does not cause undue stress to study subjects. This method is increasingly used in molecular epidemiology studies for investigating the impact of nutrition, lifestyle factors, genotoxin exposure and genotype on DNA damage, chromosome

malsegregation and cell death[12].

The buccal mucosa provides a barrier to potential carcinogens that can be metabolized to generate potential reactive products. As up to 90% of all cancers appear to be epithelial in origin, this site could be used to monitor early genotoxic events as a result of potential carcinogens entering the body through ingestion or inhalation. The Buccal Mucosa Cytome (BMCyt) assay has been used to measure biomarkers of DNA damage (micronuclei and/or nuclear buds), cytokinetic defects (binucleated cells) and proliferative potential (basal cell frequency) and/or cell death (condensed chromatin, karyorrhexis,pyknotic and karyolytic cells). As the buccal cells turn over every 7-21 days, it is theoretically possible to observe the genotoxic effects of an acute exposure approximately 7-21 days later .[12,13]

Acridine Orange (AO) is a nucleic acid selective metachromatic stain useful for cell cycle determination. It interacts with DNA and RNA by intercalation or electrostatic attraction respectively. DNA intercalated AO fluoresces green (525nm); RNA electrostatically bound AO fluoresces red (>630nm). It may distinguish between quiescent and activated, proliferating cells, and may also allow differential detection of multiple G_1 compartments. This staining may also be useful as a method for measuring apoptosis, and for detecting intracellular pH gradients and the measurement of proton-pump activity[14,15]. There are many false-positive results in micronuclei frequency as a result of using Romanowsky-type stains such as Giemsa, May-Grunwald Giemsa and/or Leishmann's which leads to inaccurate assessment of DNA damage. Romanowsky stains have been shown to increase the number of false positives as they positively stain keratin bodies that are often mistaken for micronuclei and are therefore not appropriate for this type of analysis. For these reasons, it is advisable to avoid Romanowsky stains in favour of DNA-specific fluorescent-based stains such as propidium iodide, DAPI,Feulgen, Hoechst 33258 or Acridine Orange[12].

The criterion of scoring is originally based in the described by Tolbert et al. that are intended for classifying buccal cells into categories that distinguish between "normal" cells and cells that are considered "abnormal" on the basis of cytological and nuclear features, which are indicative of DNA damage, cytokinetic failure or cell death. Therefore, some definitions of the cytological findings are: Normal "differentiated" cells have a uniformly stained nucleus, which is oval or round in shape. They are distinguished from basal cells by their larger size and by their smaller nucleus-to-cytoplasm ratio. No other DNA-containing structures apart from the nucleus are observed in these cells. These cells are considered to be terminally differentiated relative to basal cells, as no mitotic cells are observed in this population[12]. The micronuclei are round or oval in shape and their diameter should range between 1/3 and 1/16 of the main nucleus. They have the same staining intensity and texture as the main nucleus[16]. Most cells will contain only one micronuclei but two or more can be found . Baseline frequencies for micronucleated cells in the buccal mucosa are usually within the 0.5-2.5 MN/1000 cells

range. Cells with multiple MN are rare in healthy subjects but become more common in individuals exposed to radiation or other genotoxic events[12].

In humans, formaldehyde exposure is associated with an increase in the frequency of micronuclei in buccal epithelial cells[9,17], as corroborated by the results presented here. Costa et al evaluated the genetic effects of long-term occupational exposure to FA a group of 30 Pathological Anatomy laboratory workers by testing a variety of biological endpoints, cytogenetic tests (micronuclei, MN; sister chromatid exchange, SCE) and comet assay. MN frequency was significantly higher (p=0.003) in the exposed subjects (5.47+/-0.76) when compared with controls (3.27+/-0.69). Their results were in consistence with the present study [4].

Laderia et al done a study on genotoxicity biomarkers including micronuclei in buccal cells in individuals working in histopathology laboratories and exposed to formaldehyde. All genotoxicity biomarkers showed significant increases in exposed workers in comparison with controls. In buccal mucosa cells, the mean MN frequency was found to be significantly higher ($p = 0.002$) in exposed subjects (0.96±0.277) than in controls (0.16±0.058). Their results were also in consistence with the present study.[3]

Viegas et al conducted a study was carried out in Portugal, using 80 workers occupationally exposed to formaldehyde vapours: 30 workers from formaldehyde and formaldehyde-based resins production factory and 50 from 10 pathology and anatomy laboratories. A control group of 85 non-exposed subjects was considered. Exposure assessment was performed by applying simultaneously two techniques of air monitoring: NIOSH Method 2541 and Photo Ionization Detection equipment with simultaneously video recording. Evaluation of genotoxic effects was performed by application of micronucleus test in exfoliated epithelial cells from buccal mucosa and peripheral blood lymphocytes. Time-weighted average concentrations not exceeded the reference value of 0.75 ppm but ceiling concentrations were higher than reference value (0.3 ppm). The frequency of micronucleus in peripheral blood lymphocytes and in epithelial cells was

significantly higher in both exposed groups than in the control group ($p < 0.001$). A moderate positive correlation was found between duration of occupational exposure to formaldehyde (years of exposure) and micronucleus frequency in peripheral blood lymphocytes ($r = 0.401$; $p < 0.001$) and in epithelial cells ($r = 0.209$; $p < 0.01$).[1]

Burgaz et al assessed the cytogenetic damage related to occupational exposure to airborne chemicals during shoe-making and the processes in pathology and anatomy laboratories. The MN count per 3000 cells was measured in buccal smears from shoe-workers (group I, $n = 22$) exposed to mainly *n*-hexane, toluene and methyl ethyl ketone (MEK) and from anatomy and pathology staff (group II, $n = 28$) exposed to formaldehyde (FA). Eighteen male university staff were used as controls. The mean (±SD) micronuclei frequencies in buccal mucosa cells from workers in group I, group II and controls were 0.62±0.45%, 0.71±0.56% and 0.33±0.30%, respectively ($p < 0.05$ and $p < 0.05$ compared with controls for group I and group II, respectively). Their results suggested that occupational exposure to organic solvents, mainly *n*-hexane, toluene, MEK and FA, may cause cytogenetic damage in buccal cells and that use of exfoliated buccal cells seems to be appropriate to measure exposure to organic solvents [9].

Deepa Rani K et al suggested that formaldehyde is genotoxic *in vitro* in cultured mammalian cells. When it reaches nuclear DNA, it forms DNA-protein cross-links (DPX); incomplete repair of this DPX can lead to the formation of mutations, in particular chromosome mutations and micronuclei in proliferating cells [18].

Speit et al. suggested that formaldehyde leads primarily to local genotoxic effects at the site of contact due to its high reactivity. Volunteers (10 women, 11 men) were exposed to formaldehyde vapors for 4 h per day over a period of 10 working days under strictly controlled conditions. Two thousand cells were analyzed for the presence and the frequency of micronuclei per 1000 cells was determined on slides coded by an independent quality-assurance unit. No significant increase in the frequency of micronuclei was measured at any time point after the end of the exposure. Twenty-one days after the end of the exposure the frequencies were significantly lower in comparison with control. This study, indicates that FA does not induce MN in buccal mucosa cells after peak exposures up to 1 ppm and a cumulative exposure of 13.5 ppm h over 2 weeks[19]. In the present study the level of exposure of formaldehyde could not be assessed, as it required sophisticated equipment.

V. Conclusion:

The present study highlights that there is significant DNA damage in subjects exposed to formaldehyde. Micronuclei in buccal cells showed local genotoxic effects from the first contact of FA .There is a positive correlation between MN frequency (in epithelial buccal cells) and the duration of FA exposure (years of employment). Years of exposure are also influent upon the development of health effects. Appropriate protective measures have to be adopted. Protective measures include keeping FA stored in closed containers in well ventilated areas, adequate local exhaust ventilation and use of personal protective equipments.

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Rekha .V.R,etal. "Evaluation of Micronuclei in Occupational Exposure to Formaldehyde: A Study in Subjects In Pathology Laboratories." *IOSR Journal of Dental and Medical Sciences (IOSR-JDMS)*, 19(4), 2020, pp. 56-61.