MEDICINA y SEGURIDAD del trabajo

Originales

Nuclear alterations in nasal mucosa epithelial cells of students exposed to formaldehyde

Alteraciones en los núcleos de las células epiteliales de la mucosa nasal de los estudiantes expuestos a formaldehído

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Resumen

Introducción: El formaldehído es un compuesto con una amplia variedad y se utiliza comúnmente en los laboratorios de anatomía y patología. En la temperatura ambiente se volatiliza rápidamente en un gas acre sofocante. Su inhalación se ha correlacionado con la aparición de alteraciones nucleares en diferentes tejidos. El objetivo fue investigar si la exposición a este compuesto podría estar relacionado con la aparición de aspectos citotóxicos y genotóxicos en las células epiteliales nasales de los estudiantes del curso de anatomía humana.

Material y métodos: En este estudio las células nasales proclives se recogieron periódicamente de la mucosa de los 17 voluntarios de las carreras universitarias con diferentes cargas de clases prácticas en el laboratorio de anatomía, 30 y 90 horas semestrales. Las células fueron teñidas por el método de Feulgen y la morfología nuclear fue evaluada para la detección de posibles daños. Prueba post hoc de Dunn fue utilizada para el análisis estadístico. Correlación de Pearson fue realizada con los datos de sexo, edad y las respuestas del cuestionario.

Resultados: Las células epiteliales mostraron indicadores de citotoxicidad, mutagenicidad. Los estudiantes con una carga de trabajo más extensa en el laboratorio de anatomía mostraron perfil más grave con el aumento de cariorrexis ($p < 0,05$) en el tiempo. El análisis de micronúcleos mostró una diferencia entre la primera y segunda prueba ($p < 0,01$), pero no se mantiene en el tiempo. Los estudiantes con una menor carga de trabajo no presentaron diferencias en aspectos citológicas. Aunque cariorrexis estaba presente en un gran número de células, para este grupo no hubo diferencia significativa entre los intervalos de tiempo. Lo mismo se observó para cariolisis y micronúcleos ($p > 0,05$).

Conclusión: Individuos expuestos durante cortos períodos de tiempo a formaldehído están sujetos a la acción tóxica de gas. Cariorrexis fue la característica citotóxica observada con mayor frecuencia y micronúcleos mostraron un aumento entre el primer punto de tiempo. Los diferentes patrones de daños

observados entre los grupos de estudiantes sugieren que el efecto negativo de gas puede está relacionado con el tiempo de exposición.

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Palabras clave: *carcinógenos, formaldehído, mucosa nasal, mutágenos.*

Abstract

Introduction: Formaldehyde is a compound with a wide range and is commonly used in anatomy and pathology laboratories. At room temperature is quickly volatilized to a pungent and suffocating gas and its inhalation has been correlated to nuclear alterations in different tissues. We aimed to investigate whether exposure to this compound was correlated with the appearance of cytotoxic and genotoxic features in the nasal epithelial cells of students enrolled in a human anatomy course.

Material and Methods: This prospective study collected periodically nasal cells from mucosa of 17 volunteers from two different undergraduate programs with different workloads of practical lessons in an anatomy laboratory, 30 and 90 hours per semester. Cells were staining according to Feulgen method and nuclear morphology was analyzed to detect possible damage. Dunn's post hoc test was used in the statistical analysis. Pearson's correlation was performed for gender, age and questionnaire responses.

Results: Epithelial cells showed indicators of cytotoxicity and mutagenicity. Students with a more extensive workload in anatomy laboratory displayed a more severe profile with an increase in karyorrhexis ($p < 0.05$) over time. The micronucleus analysis showed difference between first and second collection ($p < 0.01$), although it was not maintained over the time. Students with a less extensive workload display no differences in most of cytological features. Despite karyorrhexis was present in a greater number of cells, for this group no significant difference was observed between any range. The same was observed to karyolysis and micronucleus ($p > 0.05$).

Conclusion: Individuals exposed for short periods of time to formaldehyde are subject to the toxic action of this gas. Karyorrhexis was the most frequently observed cytotoxic feature and micronucleus showed an increase between the first time point. The patterns observed between the student's groups suggest a negative effect due to exposure time.

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Key words: *carcinogens, formaldehyde, nasal mucosa, mutagens.*

INTRODUCTION

Formaldehyde (methanal) is the simplest and reactive aldehyde. It is a compound with a wide range of industrial applications and is commonly used in anatomy and pathology laboratories as a fixative to preserve anatomical specimens. It is also a product of normal human metabolism[1, 2]. In its natural state, formaldehyde is a gas, and due to its high water solubility, aqueous solutions of the reactant can be produced[1].

At room temperature, formaldehyde is quickly volatilised to a pungent and suffocating colourless gas with a distinct odour, which can be recognised by humans at concentrations below 1 ppm[3]. Inhalation of formaldehyde is thought to lead to deposition and/or absorption, mainly in the oral and nasal mucosa, regions that are in direct contact with the gas[4]. According to World Health Organization International Agency for Research on Cancer it is a carcinogenic product[5].

Remarkably, the cytological analysis of exfoliated cells from the nasal mucosa still constitutes an important tool to study the extent DNA damage and is also useful to detect epithelial changes. One of the reasons for the usefulness of this method is that it is easy to obtain a tissue sample, and this method yields good quality and sufficient quantities of DNA[6].

Among the nuclear aspects that are viable for analysis and interpretation, the most frequently cited phenotypes in the literature are the presence of karyorrhexis (KR), karyolysis (KL), and pyknosis (PN) (indicators of cytotoxicity), as well as the presence of a micronucleus (MN). The presence of a MN is the most widely used marker for mutagenicity[6-8].

The MN is a small cytoplasmic mass, which is microscopically visible as a circular or oval shape, and is located near the nucleus[3]. Molecularly composed of compacted chromatin, the MN results from aberrant mitosis and consists of acentric chromosome fragments, chromatids or chromosome aberrations that are induced by clastogenic or aneugenic agents[9].

The ability of formaldehyde exposure to promote the presence of a MN is recognised in the literature. However, there are still questions about the frequency and amount of MN[3]. It is important to emphasise that, although dispersed formaldehyde could be rapidly metabolised, the molecules that enter the cytoplasm of mitotically active cells could cause DNA damage, resulting in an increased frequency of MN[4]. Therefore, it is useful to perform a cell count with this marker because it is a valuable tool to detect the genotoxic potential of various chemical compounds[1, 10, 11].

Moreover, the nuclear abnormalities that characterise cytotoxicity are important markers for cell death. KR results from fragmentation and the dispersion of the nucleus in the cytoplasm. KL represents digestion of the chromatin and is easily identified as the presence of cells with no nucleus. PN is characterised by cells with small and hyperchromatic nuclei[12].

Although the focus of previous studies was to analyse the well-recognised cytological abnormalities mentioned above, variables such as exposure time, gas concentration at the time of exposure, and other related factors (for example smoking, alcohol intake, acute infections and severe allergies)[1, 3] still vary greatly among the available references. This suggests a lack of standardisation and systematisation of the collection and analysis of previous data[13]. Furthermore, most studies focused on chronic formaldehyde exposure and its effects on cytological tissues. This study aimed to analyse the presence of nuclear changes in epithelial cells from the nasal mucosa of individuals who were exposed to formaldehyde during few months.

MATERIAL AND METHODS

Study Population

The sample groups were composed of students from the biological sciences program (Group 1) and the dentistry program (Group 2). All of the students selected were enrolled in a human anatomy class. The decision to include two different undergraduate groups of students was based on the different workloads within the human anatomy discipline. In the biological sciences program, practical classes occur once a week for two hours over 15 weeks per semester, with a total of 30 hours per semester of laboratory study. On the other hand, in the dentistry group, there are three practical classes of two hours per week, also over 15 weeks per semester, with a total of 90 hours of laboratory study. Is important to emphasise that the practical lessons for both disciplines occur in the same anatomy laboratory; thus, the students come into contact with formaldehyde gas in similar surroundings. The procedures were in accordance with the ethical standards on human experimentation and were approved by the university`s ethical committee (process number: 274/10).

Questionnaire

Before the first practice session, a questionnaire was given to establish a sample profile. The questionnaire was also a tool to screen for exclusion criteria, which included: history of cancer, prior chemotherapy and/or radiotherapy, diabetes, smoking, heavy alcohol consumption, previous contact with formaldehyde, radiographic exams in the chest and neck in the last 16 days and use of anabolic steroids. The volunteers were also asked for information regarding chronic bronchitis, asthma, chronic rhinitis, nasal solution use, orthodontic treatment, and medication use.

Measurements of formaldehyde gas

The sanitary policy of the university states that measurements of toxic gases in laboratories that use formaldehyde as a fixative and preservative of specimens must be performed. The analysis was performed by an independently contracted company, and the rate of formaldehyde gas exposure in the anatomy laboratory was 0.73 ppm.

Cells collecting and microscopic analysis

The nasal mucosa cells were collected by scraping the nasal cavity with a wooden spatula that was previously moistened with saline to minimise the discomfort and to allow for proper cell collection. Different time intervals were established for this study. The first collection was performed immediately before the first practice session in the anatomy laboratory (t0), and the others were carried 14 and 56 days after the first collection (t1 and t2, respectively). The last collection was made 90 days after the last contact with the gas (t3).

Immediately after collection, the cells were dipped in 1 ml of saline and fixed with 1 ml of a methanol and acetic acid (3:1) solution. Subsequently, histologic slides were prepared by smearing. The slides were set aside for 24 hours and were then stained. The staining was performed according to the Feulgen method[12]. A total of 100 cells per slide were counted by trained investigators, which achieved a Fleiss' kappa index of 0.8203.

For the histopathological studies, the cells were analyzed to evaluate for the presence of nuclear changes including: KR, PN, KL and the presence of MN. All cells included in the count needed to present the following features: an intact and relatively flat cytoplasm, no overlap with adjacent cells, little or no debris and an intact and distinct nuclear perimeter[14]. The classification criteria for cytotoxicity features was the same as described by Tolbert et al[14] The MN were scored when they presented with four criteria: chromatin structure and colour intensity were similar to or weaker than those of the main nucleus, the borders were easily recognisable, they were «roundish», and they were in the same

cytoplasm as the main nucleus[15]. All of the slides were masked to avoid evaluator-related bias in interpretation of the results.

During the microscopic analysis, an overview was made to qualify the slides, and then, each slide was evaluated with a 40x objective to determine if any of the cellular alterations were present (Olympus AX70, Center Valley, PA, USA. Zeiss Camera Erc 5, Oberkochen, BW, Germany).

Statistical analysis

GraphPad Software (La Jolla, CA, USA) was used for data analysis and graph construction. The Dunn's post hoc test was used in the statistical analysis. The Pearson's correlation was performed for gender, age and questionnaire responses. The results were considered significant when $p < 0.05$.

RESULTS

A total of 34 students agreed to participate in the study, 17 from the sciences program (Group 1) and 17 from the dentistry program (Group 2). The questionnaire served as a tool for exclusion and provided data to establish the volunteer's profile. No volunteers reported a history of cancer, chemotherapy, radiotherapy, diabetes or use of steroids. Concerning alcohol consumption, the students reported a low frequency of consumption. For these students, if none of the other criteria prevented their participation, they were included. After analysing the answers, a total of 17 volunteers were selected to participate in the next steps.

Regarding clinical profile, groups showed similar features. Although it was detected difference in age, this aspect did not affect the results once the average was between 20-25 years, with 20 years as mean age for both groups. The data obtained from the questionnaire is presented in table I.

Table I. Questionnaire data

	Biology Students	Dentistry Students	p-value
Volunteers: n	6	11	—
Mean Age	20.5 ± 1.97	20.0 ± 5.38	0.0442*
Gender: n (%)			
Male	2 (33.3%)	2 (18.8%)	0.584
Female	4 (66.7%)	9 (81.8%)	
History of cancer	0	0	—
History of chemotherapy	0	0	—
History of radiotherapy	0	0	—
History of diabetes	0	0	—
History of rhinitis/chronic bronchitis	0	5 (45.4%)	0.1023
Smokers	0	0	—
Alcohol consumption	2 (33.3%)	2 (18.8%)	0.5467
Undergoing orthodontic treatment	3 (50.0%)	3 (27.3%)	0,6
Use of mouthwash	1 (16.6%)	1 (10.0%)	1
Use of medication (e.g., corticosteroids, anti-inflammatory, antibiotics, etc.)	3 (50.0%)	1 (9.1%)	0.2848
Subjected to any treatment during the study	2 (33.3%)	0	0.1103
Previous contact with formaldehyde	1 (16.6%)	2 (18.8%)	1
Head and neck radiographs up to 16 days before beginning study	0	0	—
Use of steroids	0	0	—

Among the nuclear aspects analyzed, figure 1 illustrates the microscopic features of the epithelial nasal cells that were considered normal (A) and cells with and with MN (B), PN (arrow, B), KL (C) and KR (D). The cytological criteria established to describe each nuclear alteration were followed, as described above. Nuclear features observed after each time were counted and the mean values are presented in table II for both groups.

Figure 1. Microscopic features of nuclear aspects in nasal epithelial cells. (A) Normal cells; (B) Micronucleus and pyknosis (arrow); (C) Karyolysis; (D) Karyorrhexis. Feulgen/Fast Green stain. Scale bar = 50 µm

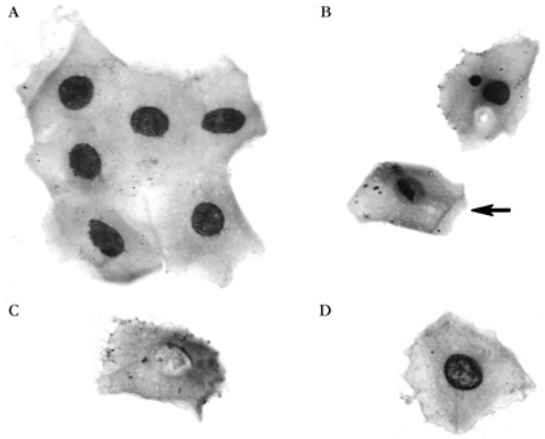


Table II. Nuclear features over time in both groups

	Biology Students		Dentistry Students	
t0	**Mean**	**SD**	**Mean**	**SD**
Normal	68.75	4.40	79.41	17.59
Karyorrhexis	12.40	3.32	6.43	3.61
Pyknosis	10.50	1.77	7.79	2.76
Karyolysis	1.01	1.41	1.19	1.50
Micronucleus	0.70	0.58	0.33	0.55
t1	**Mean**	**SD**	**Mean**	**SD**
Normal	76.88	5.94	63.75	13.64
Karyorrhexis	14.09	6.70	13.56	5.71
Pyknosis	7.92	4.01	11.66	5.01
Karyolysis	2.79	1.78	4.00	1.87
Micronucleus	0.70	0.88	1.45	0.58
t2	**Mean**	**SD**	**Mean**	**SD**
Normal	70.67	4.22	67.40	12.56
Karyorrhexis	9.89	2.47	14.95	4.98
Pyknosis	13.22	2.20	10.28	4.61
Karyolysis	3.16	1.67	1.89	2.40
Micronucleus	1.30	0.79	0.39	0.52
t3	**Mean**	**SD**	**Mean**	**SD**
Normal	72.07	6.41	77.45	15.52
Karyorrhexis	16.00	16.74	20.39	8.80
Pyknosis	15.57	4.12	4.39	4.59
Karyolysis	3.50	1.63	1.64	1.81
Micronucleus	1.36	1.03	0.07	0.15

The results obtained from the analysis and identification of nasal mucosa cytological features in the biology students (Group 1) showed that cells with a normal nucleus were the majority at all ranges defined for this study. A significant difference in abnormalities was found between t0 and t1 ($p < 0.05$), with an increase in the number of cells that were abnormal at t1. Despite the fact that KR was present in a greater number of cells, no significant difference was observed between any of the ranges. The cells identified with nuclear PN showed differences between t0 and t3 ($p < 0.05$) and t1 and t3 ($p < 0.01$). The frequency of KL was the lowest of the observed cytotoxic aspects at all tested time intervals. Although the data suggest an increase in KL over time, there were no significant differences between the time points. Similar data were obtained with regards to the presence of a MN, which was the least abundant nuclear finding in the samples (table III).

Table III. Results of Dunn's multiple comparison test performed on the data collected at t0, t1, t2 and t3 obtained from the biology students (Group 1)

	p-value summary				
Time	**Normal**	**Karyorrhexis**	**Pyknosis**	**Karyolysis**	**Micronucleus**
t0 vs t1	< 0.05*	ns	ns	ns	ns
t0 vs t2	ns	ns	ns	ns	ns
t0 vs t3	ns	ns	< 0.05*	ns	ns
t1 vs t2	ns	ns	ns	ns	ns
t1 vs t3	ns	ns	< 0.01**	ns	ns
t2 vs t3	ns	ns	ns	ns	ns

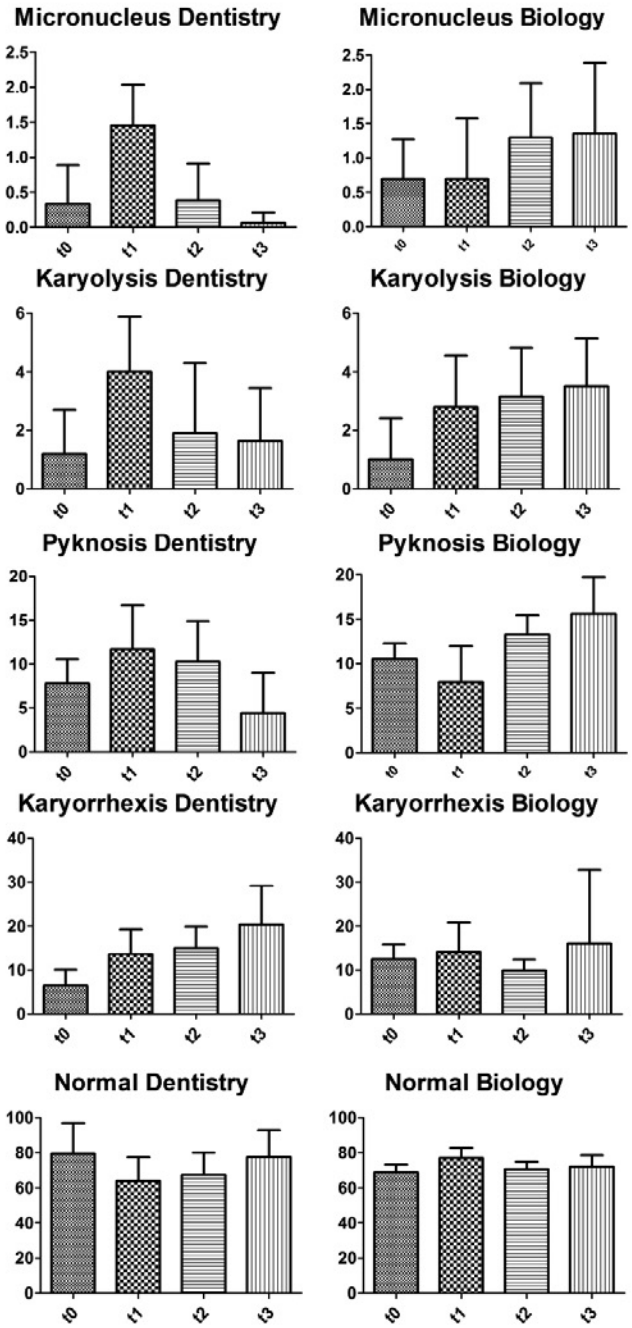
The same analyses were performed for the dentistry students (Group 2). As noted for Group 1, the nasal cells with a normal nucleus were the majority at all ranges defined for this study. However, representative differences were present in more than one-time interval. Initially, a reduction in normal cells was detected between t0 and t1 ($p < 0.01$), with a further increase between t1 and t3 ($p < 0.05$), reaching values close to those obtained in t0, before exposure to formaldehyde. KR was the most frequently observed cytotoxic feature, which was similar to the data observed in the biology students. However, a significant increase in this nuclear alteration was detected between t0 and t2 ($p < 0.05$) and t0 and t3 ($p < 0.001$). When PN was analyzed, we found a decrease between t1 and t3 ($p < 0.01$). This finding differs from that observed in Group 1. A representative increase in cells with KL appeared after the first formaldehyde exposure (t0 and t1, $p < 0.05$), and a decrease was recorded at a subsequent time (t2, $p < 0.05$). As described for Group 1, KL was also the least frequent cytotoxic nuclear feature in the microscopic findings. The MN analysis showed an increase between t0 and t1 ($p < 0.01$), although it was not maintained over the time. Still, a decrease was observed when comparing t1 and t3 ($p < 0.01$) (table IV).

Table IV. Results of the Dunn's multiple comparison test performed on the data collected at t0, t1, t2 and t3 obtained from the dentistry students (Group 2)

	p-value summary				
Time	**Normal**	**Karyorrhexis**	**Pyknosis**	**Karyolysis**	**Micronucleus**
t0 vs t1	< 0.01**	ns	ns	< 0.05*	< 0.01**
t0 vs t2	ns	< 0.05*	ns	ns	ns
t0 vs t3	ns	< 0.001***	ns	ns	ns
t1 vs t2	ns	ns	ns	< 0.05*	ns
t1 vs t3	< 0.05*	ns	< 0.01**	ns	< 0.01**
t2 vs t3	ns	ns	ns	ns	ns

The profile of cells number identified as normal and with nuclear changes at each time is presented in the graphs in figure 2.

Figure 2. Graphical representation of nuclear changes over time. The y-axis represents the number of features. The bars represent the mean amount of features observed ± the standard deviation



DISCUSSION

This study aimed to assess whether individuals exposed to formaldehyde gas for short periods of time developed damage to the epithelial cells in the nasal mucosa. We also attempted to determine if the pattern of cellular damage varies between two groups that were exposed for different amounts time. The data demonstrated that cells exposed to formaldehyde (even at low levels) showed nuclear alterations, and students with a more extensive workload in the human anatomy discipline displayed a more severe profile.

The presence of MN is believed to be one of the most sensitive tools available to search for genotoxicity induced by formaldehyde[3, 9, 16]. For biology students, no statistically significant differences were found between the frequency of MN after contact with formaldehyde. This result demonstrated that the exposure time alone was not enough to induce appearance of this specific important feature.

A different scenario was found for the dentistry students. A steep increase in the frequency of MN was detected at t1 compared to t0. This increase was followed by a gradual decrease at t2 and t3. The reduction in MN frequency was significant when comparing t1 to t3. These data suggest that the first effect was cell damage. However, after 90 days without exposure to the gas, the epithelial cells were able to recover. Furthermore, the recovery in Group 2 observed between t2 and t3 suggests that the nasal mucosa could have different response patterns depending on the severity and nature of harmful stimuli. On the other hand, individuals who are exposed to a higher burden of formaldehyde suffer sudden changes in cellular nucleus.

When analysing the frequency of KL, we observed the same tread that occurred with the MN. While Group 1 showed a gradual increase over time (remaining even after the end of gas exposure), Group 2 showed a dramatic increase at t1, followed by recovery at subsequent time points. The presence and enhancement of KL indicated cell damage, confirming the potential toxic effects of formaldehyde on the nasal mucosa[4, 6]. It also suggests that the process of toxicity could be dependent on the exposure time.

Another nuclear change, PN, has a pattern that is similar to that observed for MN and KL. However, the statistical significance appeared at different time points. In Group 1, there was a difference between t0 and t3 and a difference between t1 and t3. These data demonstrated a slow ascending effect of formaldehyde on the nasal mucosa of the subjects. On the contrary, the trend observed in the analysis of Group 2 suggest a faster negative effect, most likely associated with the greater number of hours per week that these students were exposed to the gas when, compared to biological sciences students (Group 1). At t3, we observed recovery of the normal cells levels.

With regards to KR, it is important to notice that no statistically differences were found in Group 1, while there were differences between t0 and t2 as well as between t0 and t3 in Group 2. This suggests that increased periods of exposure to formaldehyde (not only the gas concentration) are sufficient to increase the cell number with KR. Additionally, these data suggest that a residual and persistent toxic effect of formaldehyde could occur in epithelial basal cells.

The literature reports a wide variation in the incidence of MN[8]. Comparing our results with others studies, MN was detected in a higher frequency[1, 17]. This difference could be accounted for by socioeconomic and cultural differences, but the most important feature could be the lack of reliable universally accepted standards and techniques for the technical analysis of epithelial nasal cell alterations[13, 18]. Some medications and drugs have been identified as inducing the appearance of nuclear changes, specially MN. Diabetes mellitus patients using pioglitazone and glimepiride in combination showed increased frequency of MN in oral mucosa cells[19]. The same was observed in crack cocaine users[20]. Regarding nasal cells, most of studies related nuclear damage with pollution and carcinogenic compounds. In our sample, some students mentioned history of rhinitis/ chronic bronchitis, however they did not manifest the disease at the study time and did not use any medication.

These differences suggest the establishment of methodological divergence, which can influence the collection of the final results. Another limitation is that most of the previous studies were conducted with occupational exposure individuals, which is quite different from the profile of the individuals in this study. The absolute counts of MN found were close to those that were observed by Ying et al[21], ranging from 0.35 to 0.75 per sample in non-exposed individuals and from 0.75 to 1.5 per sample after a short-term exposure.

CONCLUSION

This study demonstrated that individuals exposed for short periods of time to formaldehyde are subject to the toxic action of this gas. Increases in the frequency of micronucleated cells over time reinforce the evidence of the genotoxic potential of formaldehyde in epithelial nasal cells. Moreover, the different damage patterns observed between the student groups suggested a load-dependent effect. The gas-cell interaction was affected by the duration of exposure when individuals were exposed to the same concentration of gas. However, further studies are necessary to confirm these findings, especially in for case of a short exposure.

CONFLICT OF INTEREST

Conflicts of interest: none.

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