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GENOTOXICITY BIOMARKERS AMONG FORMALDEHYDE EXPOSED SUBJECTS IN ANATOMY & EMBRYOLOGY DEPARTMENTS, FACULTY OF MEDICINE ZAGAZIG UNIVERSITY

By

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Abstract:

Introduction: A genotoxic effect of formaldehyde (FA), particularly micronucleus (MN) induction and chromosomal aberrations (CA) has been shown in several previous studies. **Aim of work:** The aim of the present study was (1): to assess formaldehyde air concentration in different areas of mortuary and museum of Anatomy& Embryology department of Zagazig Faculty of Medicine (2) to measure frequency of micronuclei and chromosomal aberrations in peripheral blood lymphocytes of the workers and staff members working in the Anatomy & Embryology department of Zagazig Faculty of Medicine as a measure of formaldehyde carcinogenicity. **Materials and Methods:** A comparative cross sectional study was carried out in Anatomy & Embryology departments of Zagazig Faculty of Medicine, where 42 subjects (occupationally exposed to formaldehyde vapors) were participated in the study and similar number of unexposed subjects was included for comparison. Exposure assessment was performed by environmental monitoring for formaldehyde concentration in the air within the mortuary and museum using a specific apparatus. Evaluation of genotoxic effects was performed by application of micronucleus test and searching for chromosomal aberrations (CA) in peripheral blood lymphocytes of the included subjects. **Results:** Time weighted average concentrations of FA (TWA8h) (measured in different

spots within the mortuary and museum) exceeded the reference value (0.75 ppm according to OSHA Scientific Committee for Occupational Exposure Limits) with mean concentration of (4.7 ppm). The frequency of micronucleus in peripheral blood lymphocytes was detected in the exposed and control group (MN mean frequency =1.4 and 0.9 respectively). Chromosomal changes were detected to a lesser extent in the two groups (mean frequency of chromosomal changes in the two groups = 0.5 and 0.5 respectively). No significant variation was proven regarding these genotoxic parameters between the two comparative groups, and also between staff members, workers of the mortuary. **Conclusion and Recommendations:** The population studied in this work is exposed to high concentration of formaldehyde for variable durations, which can be a cause of genotoxicity on the long run so, although the insignificant genotoxic parameters in our research. We recommend further investigations with broader scope, sophisticated tests and larger sample size in future human studies.

Key words: Formaldehyde; Anatomy department; Air monitoring; Genotoxicity; Micronuclei satisfaction.

Introduction

Formaldehyde (FA) is a flammable, colorless, and readily polymerized gas at room temperature (NTP, 2005). It has a pungent suffocating odor that is recognized by most human subjects at concentrations below 1 ppm (IARC, 2006). It is present in the environment as a result of natural processes and from man-made sources, and also in small quantities in every human cell (IPCS, 1989).

Occupational exposure to formaldehyde involves not only workers in direct production of FA, but also, its products and industries utilizing it (Stayner et al., 1994; IARC, 2006; Zhang et al., 2009).

The highest level of human exposure to this aldehyde occurs in occupational settings, namely, in pathology and anatomy laboratories, where FA is commonly used as a fixative and tissue preservative. Several studies consistently showed that the levels of airborne FA in anatomy laboratories exceeded recommended exposure criteria (Costa et al., 2011).

The European Scientific Committee for Occupational Exposure Limits (SCOEL) anticipated that an 8-h time-weighted average (TWA8h) exposure to 0.2 ppm FA would not be irritating and not genotoxic in humans while 0.75 ppm was a safe TWA8h in occupational setting (NTP, 2005).

International Agency for Research on Cancer (IARC), based on sufficient data, reclassified FA as a human carcinogen. (Costa et al., 2011). Previous studies reported a carcinogenic effect in humans after chronic exposure to formaldehyde, in particular an increased risk for nasopharyngeal cancer (Hildesheim et al., 2001; Coggon et al., 2003; Lubin et al., 2004).

Numerous studies have shown that formaldehyde is genotoxic and mutagenic to mammalian cells and that it induce a broad spectrum of genetic effects (Ma and Harris, 1988; IARC, 1995; Conaway et al., 1996).

The primary and direct genotoxic effect of FA seems to be the formation of DNA–protein crosslinks (DPC) in target tissues with wide evidence from both in vitro and animal studies (Shaham et al., 2003). The micronuclei test (MN) provides a reliable measure of chromosomal breakage and loss at lower cost and more easily than chromosomal aberrations (Bonassi et al, 2001).

Aim of Work

The goal of this study is to contribute to the investigation of genotoxic effects in subjects occupationally exposed to FA. Our objectives were :(1) to assess formaldehyde air concentration in different areas of mortuary and museum of Anatomy& Embryology department of Zagazig Faculty of Medicine. (2) to assess its genotoxicity biomarkers by measuring frequency of micronuclei and chromosomal aberrations in peripheral blood lymphocytes (tracing for carcinogenicity outside the portal of entry) of the workers and staff members working in the Anatomy & Embryology departments of Zagazig Faculty of Medicine.

Materials and Methods

I. Study design and setting:

A comparative cross sectional study was conducted among exposed subjects to formaldehyde in Anatomy and Embryology department of Zagazig Faculty of Medicine (randomly selected workers and staff members) compared to non-exposed administrative workers over the period of 8 months (April 1st to December 30th 2013).

II. Study sample:

First we made a random selection between the departments of high FA exposure during their work courses in Zagazig Faculty of Medicine (Anatomy and Embryology, Histology, Pathology, Forensic, and Histopathology

departments) that resulted in selection of Anatomy and Embryology department.

The working force of Zagazig Anatomy and Embryology departments at the time of the study was 56 subjects and excluding staff members with no current exposure to formaldehyde as professors and assistant professors, secretary employees, subjects who were absent in leaves or abroad travelers. Finally, 45 persons who were exposed currently to FA (staff instructors, producers of FA preserved organs in jars, postgraduate lecturers and workers in the mortuary and museum) were invited to participate in the study. Three smokers were excluded later ending in 42 subjects finally enrolled in the study and a matched 42 control subjects were chosen subsequently from administrative workers of Zagazig University.

III. Study Population:

1- Exposed group:

- **Inclusion criteria:** Subjects working in Anatomy and Embryology departments of Zagazig, faculty of Medicine wether staff members or workers with at least one year duration of work and exposure to FA were invited to participate in the study
- **Exclusion criteria:** those who had history of co-exposure to other chemicals, less than one year of work, smokers or those with alcohol positive history.
- Response rate was 93% after exclusion of three smokers from the study group.

2- Non-exposed group (Control):

A matched nonsmoking group of 42 subjects with no history of specific FA or chemicals exposure were selected from administrative workers of Zagazig University.

IV. Study Methods

1- Structured questionnaire inquiring about personal data, medical history and lifestyle factors for all studied individuals, as well as information related to work duration, practice and exposure was distributed for all the participants.

2- Environmental monitoring for of FA exposure: occupational exposure of formaldehyde air concentration was assessed in 6 places within the mortuary and museum of the Anatomy department (teaching class, dissection halls, around cadaver, museum, cadaver keeper, and staff room).

Exposure assessment was performed by obtaining air samples by using a specialized instrument of flow-adjusted pump containing a specific formaldehyde absorbent in distilled water obtained from the Egyptian National Research Centre (air pollution department). Formaldehyde concentration calculated (by a special equation) for absorbed formaldehyde in certain time (Stern, 1986).

Equation of HCHO (formaldehyde) Gas Analysis and Calculation of its concentration:

Absorbance Factor from standard curve ml equivalent ((10ug HCHO/ ml) sample volume 1000

Standard curve factor and absorbance:

Method: dilute 1ml of stock standard solution(diluted 2.7 ml of 35-37% formaldehyde into 1litre distilled water) into 100 ml by distilled water and prepare series of solution 0.5,1,3,5,7 and diluted into 50 ml by absorbing solution (0.5 gm MBTH 3-METHYL-2 - B E N Z O T H I O Z O L O N E HYDROZONE HYDROCHLORIDE in litre distilled water) then add 2ml oxidizing reagent solution (1.6 gm sulphoric acid and gm ferric chloride in 100ml distilled water) then allow 12 minute before measuring absorbance at wavelength 628nm then plot calibration curve and determine factor according to Stern, 1986.

Adsorbed formaldehyde was collected by the researchers of Occupational Medicine department guided by the anatomy researcher and FA analysis was done by specialists in the Air Pollution department of the Egyptian National Research Centre.

3- Biological monitoring (genotoxicity tests):

To evaluate the genotoxic effect of FA occupational exposure, biomarkers of genotoxic effect were studied, namely micronuclei and chromosomal aberrations.

Method: 4ml of heparinized venous blood were collected from every one of the studied group by a nurse from the hospital under aseptic conditions where, 2ml were sent for pathological detection of chromosomal changes and the other 2ml were sent for histological detection of micronuclei to be processed within 6hours. All samples were coded and analyzed under blind conditions

A- Method of Chromosomal analysis:

Samples collected in lithium heparinized tubes, coded and sent to the laboratory. Lymphocyte cultures were set up by adding 0.5 ml whole blood to 4.5 ml RPMI 1640 medium supplemented with L-glutamine, 15% heat- inactivated fetal calf serum and 1% antibiotics (penicillin and streptomycin). Lymphocytes were stimulated by 1% phytohaemagglutinin (all obtained from Gibco laboratories) and incubated for 72 h at 37°C. Two cultures per subject were established. At 72 h of incubation, the cultures were harvested. Colcemid (10 ug/ml) was added to the culture and left for 30 min at 37°C then treated with prewarmed hypotonic solution (potassium chloride,5.59g/L) for 30 min at 37°C. Cells were centrifuged thereafter and a fresh, cold fixative solution (3:1 methanol: acetic acid) was added drop by drop slowly. The fixation step was repeated twice and the resulting cells were resuspended in a small volume of fixative solution and dropped onto clean slides from a height of about 40-60 cm. The slides were allowed to age for at least one day at 37°C.

- Finally the slides were stained with 10% Giemsa in phosphate buffer (pH 6.8) for 10 min. Slides were scanned under a light microscope to locate the chromosome spreads, which were then examined under high power oil immersion x100 objective. At least 20 metaphases were examined.

- Chromosome analysis was done using an automated karyotyping system, Fluorescence microscope (Olympus, BX40) and a computerized image analysis system (Morphostar Genetics workstation), Karyotyping was done according to the International System for Human Cytogenetic Nomenclature .

B- Method of micro-nucleus (MN) test: micronuclei are a marker of phenotypic susceptibility to cancer that received decisive support from mutagens sensitivity studies (Bonassi et al., 2001).

- Lymphocyte cultures and MN analysis:

Blood samples were obtained from each subject by venipuncture in heparinized vacutainers, coded and sent within maximum 24 h to the laboratory where they were processed. Lymphocyte cultures were set up by adding 0.5 ml whole blood to 4.5 ml RPMI 1640 medium supplemented

with 15% heat-inactivated fetal calf serum, 1% antibiotics (penicillin and streptomycin) and L-glutamine. Lymphocytes were stimulated by 1% phytohaemagglutinin (all obtained from Gibco laboratories) and incubated for 72 h at 37°C. Two cultures per subject were established. A final concentration of 6 μg/ml cytochalasin B (Sigma, St Louis, MO) was added to the cultures 44 h later to arrest cytokinesis. At 72 h of incubation, the cultures were harvested by centrifugation at 800 r.p.m. for 8 min and treated with a hypotonic solution (2–3 min in 0.075 M KCl at 4°C). Cells were centrifuged thereafter and a 3:1 (v/v) methanol: acetic acid solution was gently added. This fixation step was repeated twice and the resulting cells were resuspended in a small volume of fixative solution and dropped onto clean slides. Finally the slides were stained by 10% Giemsa in phosphate buffer (pH 6.8) for 10 m and scored. To determine the frequency of binucleated cells with micronuclei and the total number of MN in lymphocytes, a total of 1000 binucleated cells with well-preserved cytoplasm (500 per replicate) were scored per subject on coded slides. This is the number of cells usually scored in most laboratories. For the scoring of micronuclei the following criteria were adapted from Fenech et al. (1999)

1. The diameter of the MN should be less than one-third of the main nucleus.
2. MN should be separated from or marginally overlap with the main nucleus/nuclei as long as there is clear identification of the nuclear boundary.
3. MN should have similar staining as the main nucleus/nuclei.

Data management:

Data was coded and statistically analyzed using SPSS version 19 (IBM, 2010). Comparison between group means was done using Student›s t test; comparison between categorical variables was done by $\chi 2$ test. A p-value < 0.05 was considered statistically significant.

Consent:

Informed consent was obtained from those who accepted to participate in the study.

Ethical Approval:

Permission was obtained from the head of department of Anatomy and Embryology. Proposal acceptance was obtained from the Research Ethics Committee (REC) in Zagazig Faculty of Medicine. Moreover, informed consent was obtained from all participants.

Results

Table1: Age, sex and duration of work distribution of the studied subjects.

	Exposed (n= 42)	Control (n= 42)	χ^2	P value
Age (years)				
≤ 30years	14 (33.3%)	17 (40.5%)	0.46	0.497
> 30 years	28 (66.7%)	25 (59.5%)		
Gender				
Female	27 (64.3%)	30 (71.4%)	0.49	0.483
Male	15 (35.7%)	12 (28.6%)		
Duration of work				
≤ 10 years	16 (38.1%)	17 (40.5%)	0.05	0.823
> 10 years	26 (61.9%)	25 (59.5%)		

This table showed that both exposed and control groups are matched as regards to relevant socio-demographic characteristics (no significant difference).

Table (2): Formaldehyde air concentration in different sampling places within the mortuary and museum:

Site of the sample	FA exposure level (TWA_{8h}) * (ppm)	(USA-OSHA) Concentration in ppm
Teaching class 2	11.1	TWA8h 0.75
Dissection halls	6.9	
Around cadaver	5.6	
Museum	1.8	
Cadaver keeper (formalin basin)	1.6	
Staff room	1.5	

This table showed that, all measured Formaldehyde concentrations in different locations within Zagazig Anatomy &Embryology departments exceeded the OSHA permitted level with highest level at the teaching class 2.

Table (3): Frequency of micronuclei (MN) and chromosomal aberrations (genotoxic parameters) in the studied population

Test	Exposed (n=42)	Control (n=42)	t-test	P value
Frequency of MN/1000 cells Mean ± SD	1.4± 1.4	0.9±0.7	1.81	0.07
Frequency of chromosomal aberrations (CA) scored in 100 metaphase /cell Mean ± SD	0.5 ± 0.4	0.5 ± 0.5	0.513	0.610

Table 3 showed insignificant difference between exposed and control groups as regards to some genotoxicity biomarkers (MNs and numerical or structural chromosomal changes).

Table (4): Comparison between frequency of micronuclei (MN) and chromosomal changes within the exposed group according to nature of work.

Test	Workers (n=5) Mean± SD	Doctors (n=37) Mean± SD	T-test	P value
Frequency of MN Mean ± SD	1.3 ± 0.49	1.4 ± 1.5	-0.149	0.882
Frequency of chromosomal aberrations scored in 100 metaphase /cell Mean ± SD	0.19 ± 0.39	0.61 ± 0.4	-1.97	0.06

This table showed insignificant differences between staff doctors and workers regarding formaldehyde genotoxicity biomarkers.

Discussion

Formaldehyde using in the preservation of animals and human specimens has been a common practice for centuries. In medical colleges, cadavers for gross anatomy laboratories are usually prepared by using formaldehyde as embalming fluid which is carcinogenic to humans (Ahmed, 2011). In the presence of cadavers and during the process of dissection, formaldehyde vapors are emitted from the cadavers, resulting in the exposure of medical students and their instructors to elevated levels of formaldehyde in the laboratory (Takayanaqi et al., 2008). Formaldehyde is suspected to have genotype effects and has been classified by IARC as group I human carcinogen (IARC 2006). Recently the classification has been expanded with FA causing leukemia (IARC 2012). Genotoxicity biomarkers analysis such as Micronucleus formation test "MNT", SCE, NBUD … etc. is used to reveal if FA exposure can lead to genotoxicity and hence can cause cancer. So, in this study we used conventional cytogenetic analysis and MNT to study the genotoxic effect of FA on exposed subjects working in Anatomy department in Zagazig University. The "Cytokinesis Block Micronucleus Test" CBMN is also a simple practical low cost screening technique that can be used to detect changes in DNA, it can lead to diseases especially cancer so it is considered preventive and important managing measure for exposed personnel not only to FA but to other genotoxic agents as well.

In this study, the air concentration of formaldehyde in almost all areas of Anatomy & Embryology department (Table 2) was found to be much higher than OSHA's Permissible Exposure Limit (0.75 ppm), especially in the second teaching room, dissection halls, area around cadavers and museum as follows. These results are in good agreement with previous ones reported by Akbar-Khanzadah et al., 1994 who evaluated formaldehyde exposure in a group of 34 subjects in a gross anatomy laboratory and reported that TWA8h concentration of formaldehyde ranged from 0.07 to 2.94 ppm during dissecting operations and reported that 31.7% of the subjects exceeded the 8-h TWA action level of 0.5 ppm set by the OSHA. In another study by Akbar-Khanzadah and Mlynek, 1997, it was reported that airborne concentrations of formaldehyde in the gross anatomy

department was varying between 0.59 and 1.72 ppm. Moreover, a previous study assessed formaldehyde exposure in Anatomy Museum during some wet specimen activities reported 0.140 ppm formaldehyde level for area samples collected for short time (Burroughs et al., 2006).

The higher level of formaldehyde air concentration reported in this study in Anatomy department compared to other studies could be explained by the fact that measurements in this study were carried out in the presence of greater number of uncovered cadavers during condensed revision of students for final practical examination. In addition, there was insufficient ventilation system in general in the form of improperly distributed and may be unworked suction devices. This large number of cadavers and illustrating jars were brought specially for the purpose of students› revision while there was clear shortage of them all over the academic teaching year during the practical seminars of students due to financial causes, so measured FA air concentration may be adherent to a short time .

As mentioned before by (IARC 2006, Pyatt et al., 2008), the health effects (cancer) linked to FA exposure are more related with peaks of high concentrations than with long time exposure at low levels. Moreover, exposures of short duration (peaks) are of special concern, because they produce an elevated dose rate at target tissues and organs, potentially altering metabolism, overloading protective and repair mechanisms and amplifying tissue responses (Smith, 2001, Preller et al., 2004).

Teaching class 2 has the highest formaldehyde level as it lies inside the mortuary itself and exposed to more formaldehyde concentration through natural inward air draft moving formaldehyde inside the room with absent windows and unworked air suction devices . Also formaldehyde concentrations in areas closer to the cadaver were higher than other areas. The relatively higher concentrations of formaldehyde in these areas can be explained by the diffusion of formaldehyde with no doors while the concentration increased relatively beside the cadavers' keeper (tightly closed) and the in-mortuary staff room (closed door).

In this study, the frequency of micronucleus formation and chromosomal alterations showed

non-significant difference between exposed and control groups (Table 3), and no definite association was detected between unpermitted FA exposure and genotoxic biomarkers in contrast to many other studies that found significant different genotoxicity parameters between such comparative groups (Bonassi et al., 2001, Carina , 2011).

This controversy may be apparent (unreal) and different outcome may result from:

1. Difference in sensitivity of the techniques used
2. Difference in duration of exposure, concentration of the FA (Bonassi, 2001)
3. Age, sex, tobacco smoking and alcohol consumption may play role in other studies with unmatched groups (Carina et al., 2011; Jasmin et al., 2011).
4. Different populations may have different genetic polymorphism of the formaldehyde dehydrogenase (FDH) (the main enzyme catalyzing FA) or a different susceptibility to FA but it was excluded by other as Zeller et al., 2011.

Formaldehyde toxicokinetics published by (Quievry and Zhitkovich 2000) can also explain the insignificant difference between the two comparative groups as:

1. High water solubility and reactivity of formaldehyde means that airborne inhalation of FA is absorbed mainly in the upper airways so, its inhalation may not increases its concentration in the blood. So, several recent studies using labeled FA (13CD2) did not find DNA adducts
2. The nasal epithelium is an efficient barrier as Neuss et al. 2010 proved; FA was not considered to reach the internal organs or the blood compartment in rats and monkeys. Also, Lu et al., 2012; Moeller et al., 2011; Swenberg et al., 2011 and many recent toxicokinetic studies detected no exposure-dependent FA–DNA adducts outside the portal-of-entry area and reported that, FA–DNA adducts at distant sites were due to endogenously generated FA (Neuss et al., 2010)
3. The half-life of FA in blood is about 1.1 min and it is electrophilic so, reacts with DNA, RNA and proteins forming reversible adducts or irreversible cross-links.

Nevertheless, 1 % of the inhaled dose may be delivered to the blood compartment, the delivered dose would be metabolized in the blood compartment, which is a fast process and part of the delivered dose would react with blood proteins, including Hgb with valine as one of the binding sites and no accumulation is considered to occur over long-term exposures due to the fast metabolism of FA in blood and the reversible nature of the reaction products (methyl derivatives) with amino, hydroxyl and thiol groups in proteins. Even secondary reactions may also be reversible, as shown by DNA-protein crosslinks (DPX) removal in human lymphocytes due to spontaneous hydrolysis.

In the same line, many other researchers obtained nearly similar outcomes to ours as Orsière et al., 2006 who detected higher frequency but non-significant increase of peripheral lymphocytes nuclear changes and chromosomal aberrations between exposed anatomy and pathology lab workers and control subjects. Also Simon et al., 2010 found that FA did not induce any genotoxic effect on broncho alveolar lavage cells and blood lymphocytes of rates by MNT and comet assay. Also Jasmin et al., 2011 found that inhalation of formaldehyde did not lead to genotoxic effects on blood lymphocytes and nasal mucosa cells, also did not lead to alteration of dependent formaldehyde dehydrogenase or other genes expression in microarray analysis in 81 non-smoker males in contrast to Gunter Speit et al., 2009 who concluded that human cell line 549A don't develop adaptive protection against the genotoxic action of FA and neither metabolic inactivation of FA nor their repair of FA induced DPX seem to be enhanced in cells pre-treated with FA. Significant FA effects were reported also by Carina et al., 2011 who found significant difference in MN and other genotoxocity biomarkers in peripheral blood lymphocytes and buccal epithelial cells between exposed histopathology laboratory workers and in controls. They also found significant increase in these markers with elder age females and with alcohol consumption. Viegas et al., 2010 also reported significant difference of peripheral lymphocyte genotoxicity markers between anatomy and pathology staff and control subjects. Chromosomal aberrations and apoptosis indicating increased cancer risk in subjects occupationally

exposed to formaldehyde was detected by Matyas et al., 2010.

Always it was expected but no significant difference was detected between staff instructors and workers (Table 4) which can be explained by free diffusion of FA in air affecting all working personnel in the mortuary even with no direct FA contact and no special exposure to staff members.

Conclusion and Recommendations: The population studied in Anatomy and Embryology departments is exposed to high concentrations of formaldehyde for variable durations, which can be a cause of genotoxicity on the long run .In this research work, insignificant genotoxic parameters were reported which needs further investigations with broader scope, sophisticated tests and larger sample size in future studies. Formaldehyde exposed workers should stick to safety measures that can protect them from the possible harmful effect of FA (avoiding long and unpermitted exposure, wearing protective masks & gloves and organizational changes in the form of increasing ventilation windows and suction devices plus separating teaching classes from the mortuary) . Another way is to find out safe Formaldehyde substitutes which must be tried and retested for safe use as preservatives. Alternatives to Formaldehyde are important but researchers found that alcohol-based fixatives had positive and negative attributes and environmental drawbacks. Cathy et al, 2011 tested 3 new cross-linking (F-Solv [Adamas, Rhenen, the Netherlands]) and non–cross-linking (FineFIX [Milestone, Bergamo, Italy] and (RCL2 [Alphelys, Plaisir, France]) alcohol-based fixatives for routine staining in comparison with neutral buffered formalin (NBF) as the "gold standard". None was overall comparable to NBF with regard to microscopy, morphologic evaluation, and immunohistochemical studies.

Also we have to point to the importance of gene polymorphism study as (FDH) as it is the key enzyme in the FA metabolism for more precise tracing of carcinogenicity of FA in human.

Conflict of interests

Authors have declared that no conflict of interests exists.

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