Evaluation of Genomic Damage in Peripheral Lymphocytes from Occupationally Exposed Anesthetists: Assessment of the Effects of Age, Sex, and GSTT1 Gene Polymorphism

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Title: Evaluation of genomic damage in peripheral lymphocytes from occupationally exposed Anaesthetists: assessment of the effects of age, sex and *GSTT1* gene polymorphism.

Authors: Alfredo SANTOVITO, Piero CERVELLA, and Massimiliano DELPERO

University of Turin, Department of Life Sciences and Systems Biology, Via Accademia Albertina n. 13, 10123 Torino (Italy)

Running Title: **Genotoxicological monitoring of exposed Anaesthetists.**

KEYWORDS**:** CA; SCE; GSTT1; Anaesthetic gases; Occupational exposure.

Corresponding Author:

Alfredo SANTOVITO

Department of Life Sciences and Systems Biology

Via Accademia Albertina n. 13

10123 – Torino (Italy)

Tel.: +39-0116704554

Fax: +39-0116704508

e-mail: alfredo.santovito@unito.it

ABSTRACT

Occupational exposure to anaesthetic gases is one of the major hazards ~~of the~~ to healthcare personnel. We evaluated the cytogenetic effects of chronic exposure to low concentrations of anaesthetic gases in operating theatres. The study included 21 anaesthetists and 21 control subjects who matched in age and gender. Chromosome Aberrations and Sister Chromatid Exchanges assays were performed. All subjects were also genotyped for *GSTT1* gene polymorphisms.

Significant differences were found between exposed and controls in terms of Sister Chromatid Exchanges frequency ($P = 0.001$) and Replication Index value ($P = 0.005$), but not in terms of Chromosome Aberrations ($P = 0.201$) and aberrant cells ($P = 0.227$) frequencies. Regression analyses indicated that age and the years of employment did not influence the level of chromosomal damage in both groups. Finally, among anaesthetists, *GSTT1* null individuals showed a significant higher frequency of Sister Chromatid Exchange with respect to *GSTT1* positive subjects.

INTRODUCTION

Occupational exposure to anaesthetic gases, such as ethylene oxide, with potential mutagenic and carcinogenic capacity, is one of the major hazards ~~of the~~ to healthcare personnel. Although healthcare workers are exposed to low levels of pollutant, the chronic exposure to low concentrations of anaesthetic gases may result in serious disorders (Guirgius et al., 1990; Boivin, 1997; Sessler, 1997), depending on the type of gas used, the length of exposure and the gas concentrations (ISSA, 2002). For example, ethylene oxide and formaldehyde, used for sterilization, are well-known human carcinogens and are related to an increase of both chromosome aberrations (CAs) and sister chromatid exchanges (SCEs) among exposed subjects (IARC, 1988; Santovito et al., 2011).

To minimize the risk of occupational exposure to ethylene oxide, the Occupational Safety and Health Administration recommend threshold limit value time-weighted average exposures for an 8-h work of 1 parts per million (OSHA, 2009). Nevertheless, occupational exposure to low concentrations of anaesthetic gases was found to be associated with an increased risk for infertility (Rowland et al., 1992) and abortion (Boivin, 1997). On the other hand, genotoxicity related to anaesthetic gases exposure is controversial. Indeed, although in the lymphocytes of exposed subjects some cytogenetic studies evidenced an increase of SCEs (Hoerauf et al. 1999), CAs (Rozgaj et al. 2001) and micronuclei (Sessler, 1997; Hoerauf et al., 1999*;* Rozgaj and Kasuba, 2000; Rozgaj et al., 2001) other studies failed to find it (Georgieva et al., 1993; Rozgaj et al., 2001). These discrepancies could probably be due to the different exposure conditions and to the presence of other confounder factors, such as smoking habits, not always well taken into account.

The aim of the study was to evaluate the eventual genotoxic damage of non-smoker anaesthetists chronically exposed to low doses of anaesthetic gases by SCEs and CAs assays. SCEs are the result of interchanges between DNA replication products at homologous chromosomal loci (Knudsen and Hansen, 2007) and are induced by those agents forming covalent adducts to DNA or otherwise interfere with DNA metabolism and repair. Chromosome aberrations reflect damage that occurred during the G1 phase in regions that have not undergone repair or have evolved to a re-arranged element. CAs are breaks, acentric fragments, rings, dicentrics and interchromosomal exchanges which are often unstable aberrations and will lead to cell death during proliferation (Garcia-Sagredo, 2008). Generally, SCEs analysis represents a more sensitive test, particularly for S phase-dependent agents (e.g. alkylating agents), allowing for the detection of genotoxic effects at much lower concentrations than those required to induce chromosomal aberrations (Chia and Lee, 2001).

Finally, some alkylating agents, like ethylene oxide, are principally metabolyzed by conjugation to glutathione by glutathione *S*-transferase T1 (GSTT1). *GSTT1* gene is polymorphic in humans for a deletion of a segment of DNA that results in the absence of protein synthesis and consequent reduced detoxification of xenobiotics in homozygous individuals. This deletion polymorphism for the *GSTT1* gene has been found to be associated with the development of some types of cancer (Bajpai et al., 2007; Cha et al., 2007), as well as to an increased susceptibility to DNA damage (Palma et al., 2007). On the basis of these assumptions we decided to evaluate the relationships between the *GSTT1* gene polymorphisms and the levels of genomic damage measured by SCEs and CAs assays.

MATERIALS AND METHODS

Study population

The exposed group comprised 21 physicians (15 males and 6 females) working as hospital surgeon anaesthetists. The anaesthetists were exposed to different chemicals, mainly anaesthetic and sterilizing gases such as ethylene oxide and formaldehyde. All exposed subjects used complete protective equipment, according to the Italian guidelines and were routinely tested for urinary and blood drugs concentrations. The lack of data on individual exposure doses of anaesthetics is because the majority of the workers enrolled in this study are exposed to a variety of different gases and did not use a dosimeter. For this reason, anaesthetic gases exposure was considered as being the time (in years) the worker was exposed to different gases. Hence, we are interested to discover the extent of chromosome damage on peripheral lymphocytes of anaesthetists.

The control group consisted of 21 unexposed healthy individuals (13 males and 8 females) belonging to the administrative staff and working in the same hospital without any work-related exposure to hazardous agents. All the subjects of both groups lived in the same urban area.

The procedures followed in this work were approved by the local responsible committee on human experimentation and have been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. All the participants were healthy volunteers and received information about the study and gave their written consent. Prior to blood collection, each individual was extensively interviewed by a specialized physician who filled in a structured questionnaire specifying gender, date of birth, smoking status, dietary habits, alcohol consumption, work-related exposure to hazardous agents, previous exposure to diagnostic X-ray, and use of therapeutic drugs. In our sample we exclusively considered individuals that did not

smoke nor consume drugs or alcohol and were not subjected to diagnostic examinations for a period of at least two years prior to the analysis.

Cytogenetic analysis

Blood sample collection, cell cultures, SCEs and CAs assays were conducted following protocols previously published (Santovito et al., 2014). In order to determine the number of SCE/cell for each subject we scored 50 well-spread second-division metaphases containing 46 chromosomes. A total of 100 cells from each donor were scored for the determination of the replication index (RI), calculated according to the following formula: $RI = (M_1 + 2M_2 + 3M_3)/N$, where M_1, M_2 and M_3 represent the number of cells undergoing first second and third mitosis and N is the total number of scored metaphase (NSM).

For CAs assays, a total of 200 well-spread metaphases were analysed for the following categories of CAs: chromatid breaks (B'), chromosome breaks (B"), dicentrics (Dic), acentric fragments (AF), and rings (R). Gaps (a-chromatid lesions) were not scored as CAs. Cells containing any type of chromosomal aberrations were scored as cells with aberrations (CAB).

DNA extraction and GSTT1 genotyping

To extract genomic DNA we used the Chelex solution protocol as described by Walsh et al. (1991). *GSTT1* genotypes were determined by polymerase chain reaction (PCR). *GSTT1* was amplified using primers corresponding to the 3' coding region of the human cDNA, 5'-TTCCTTACTGGTCCTCACATCTC-3' and 5'-TCACCGGATCATGGCCAGCA-3'. In addition, as internal control, a fragment of the β-globin gene was co-amplified using primers 5'-

CAACTCATCCACGTTCACC-3' and 5'-ACACAACT-GTGTTCACTAGC-3'. PCR reactions were carried out in a total volume of 25 μl containing 10 ng of DNA (template), with a final concentration of 1X Reaction Buffer, 1.5 mM of MgCl2, 5% of DMSO, 250 μM of dNTPs, 0.5 μM of *GSTs* and β-globin primers, and 1U/sample of Taq DNA polymerase (Fischer, U.S.). In the thermocycling procedure, the initial denaturation at 95°C for 5 min was followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min before a final extension at 72°C for 10 min. PCR products were separated by electrophoresis on a 3% agarose gel and visualized by ethidium bromide (250 ng/ml) staining. The expected sizes of the amplified *GSTT1* and β-globin products were 480 and 110 bp, respectively. Genotypes with homozygous deletion of the *GSTT1* gene are identified as "*GSTT1*-null", whereas genotypes having at least one copy of the gene are "*GSTT1*-positive".

Statistical analysis

Statistical analysis was assessed using the SPSS software statistical package programme (version 21.0, Inc., Chicago, USA). Non-parametric Wilcoxon Mann–Whitney *U* test was used to evaluate statistical differences between groups in terms of mean age and years of employment and the frequencies of CAs and SCEs. Multiple regression analysis was used to evaluate the influence of age and years of employment on SCEs and CAs frequencies. All *P*-values were two tailed and the level of statistical significance was set at $P<0.05$ for all tests.

RESULTS

Study population

Demographic characteristics of exposed and control populations involved in the study are reported in Table 1. Twenty-one anaesthetists (15 males and 6 female, mean age 35.524±4.332) and 21 control subjects (13 males and 8 females, mean age 35.857±7.059) were recruited in this study.

No significant differences were found between and exposed and controls in terms of mean age (*P* = 0.984) and mean years of employment (*P* = 0.687).

SCEs assay

Results of SCEs assay are shown in Table 2. Significant differences were found between exposed and controls in terms of SCEs frequency (*P* = 0.001) and RI value (*P* = 0.005). In both groups, the level of SCEs frequency did not correlate with years of occupational exposure (*P* = 0.963 and *P* = 0.494 for exposed and controls, respectively) and with age (*P* = 0.789 and *P* = 0.289 for exposed and controls, respectively) (Table 4). Similarly, no statistically significant differences were observed in each group between genders (*P* = 0.249 and *P* = 0.273 for exposed and controls, respectively).

Cells with 10 or more SCEs were defined as high frequency cells (HFCs) according to Carrano and Moore (1982). Individuals for whom more than 6 cells were detected that contained more than 10 SCEs were classified as high frequency individuals (HFIs). In the total sample 12 subjects were identified as HFIs among exposed and 7 among controls. The individuals Non-HFIs were 9 and 14

for exposed and controls, respectively. HFIs showed a significant higher value of SCEs/NSM with respect to Non-HFIs in both anaesthetists and controls (Table 2).

CAs assay

Results of CAs assay are shown in Table 3. We found 4 types of aberrations: chromatid breaks, chromosome breaks, dicentrics and acentric fragments, whereas rings were not found. The most frequent types of aberration were chromatid and chromosome breaks. No significant differences were observed between exposed and controls in the frequency of CAs/NSM (P = 0.201). Among exposed individuals, the level of chromosome damage did not correlate with years of professional exposure (P = 0.229). Similarly, no correlation between CAs and age was observed in both anaesthetists and controls (P = 0.381 and P = 0.702 for exposed and controls, respectively). Finally, no significant gender differences were observed in both groups (P = 0.174 and P = 0.570 for exposed and controls, respectively).

***GSTT1* gene polymorphism**

Among anaesthetists, 15 individuals resulted *GSTT1* positive (71%, mean age 35.600±4.626) and 6 (29%, mean age 35.333±3.882) were reported as *GSTT1* null genotypes. In the controls group the *GSTT1* positives were 14 (67%, mean age 36.429±6.745), whereas the *GSTT1* null genotypes were 7 (33%, mean age 34.714±8.077) (Table 1).

Among exposed, statistically significant differences in terms of SCEs frequency were found between subjects *GSTT1* positive and those with *GSTT1* null genotype, although with a borderline p-value (P = 0.046). *Vice versa* no significant differences were found between groups in terms of

CAs (P = 0.715) and CAB (P = 0.498) frequencies. In the control group no significant differences were found in terms of SCEs (P = 1.000), CAs (P = 0.457) and CAB (P = 0.457) between *GSTT1* positives and subjects with *GSTT1* null genotype (Table 2).

DISCUSSION

Some cytogenetic studies have proven an increased number of SCEs, CAs and micronuclei among anaesthetists and other hospital workers (Hoerauf et al., 1999; Wiesner et al., 2008; Rozgaj et al., 2001). Nevertheless, other studies resulted to be ambiguous (Bozkurt et al., 2002; Wiesner et al., 2008), probably because of different sample size, different exposure conditions and because some confounder factors, such as smoking habits, were not always properly taken into account. In our sample we exclusively consider subjects that were non-smokers and that were not professionally or occasionally exposed to X-ray.

Although other authors demonstrated a significant increase of CAs among anaesthetists (Rozgaj et al., 2001) in the present study no chromosomal damage, in terms of increase of the CAs frequency, was observed (Table 3). In contrast to other published studies (Husum et al., 1983; Sardas et al., 1992 (Hoerauf et al. 1999), we observed a significantly higher frequency of SCEs among anaesthetists. The exposure to anaesthetic gases appeared to influence also the lymphocyte replication capacity, as showed by the significant lower RI value among exposed ($P = 0.005$) (Table 2).

Differently from what was observed for SCEs, increased frequency of CAs is recognized as a potential predictor of cancer (Bonassi et al., 2000, 2004). Nevertheless, damage is a consequence of the equilibrium between damage infliction and repair. In this scenario, the higher SCEs rate recorded among occupationally exposed subjects could be considered as a signal suggesting potential defects in DNA repair processes (Garcia-Sagredo, 2008).

It was suggested that cells with a high frequency of SCEs represent a long-living subset of lymphocytes that accumulated SCE-inducing lesions over time. Therefore, the evaluation of HFCs and HFIs seems to be a useful tool for assessing the effect of chronic exposure to genotoxic agents (Bozkurt et al., 2003). The higher frequency of SCEs recorded among HFIs seem to indicate the presence of a subset of individuals more susceptible to genomic damage resulting from daily environmental exposure.

In agreement with what was observed in subjects occupationally exposed to formaldehyde (Santovito et al., 2011), in our samples the length of the exposure did not influence the level of DNA damage. It could be explained by the fact that during chronic exposure part of the chromosomal aberrations can be eliminated in vivo by the death of lymphocytes. Similarly, according to published data (Anderson et al., 1993; Kašuba et al., 1995), results of our study indicated that the age did not seem to influence the levels CAs and SCEs in both exposed and control subjects (Table 4).

Finally, individuals enrolled in this study were genotyped for *GSTT1* xenobiotic metabolizing enzyme, representing biomarkers of individual susceptibility. Among anaesthetists, *GSTT1* null individuals showed a significant higher frequency of SCEs with respect to *GSTT1* positive subjects. Alkylating agents, like ethylene oxide, are principally metabolized by conjugation to glutathione by *GSTT1*. It is plausible that carriers of *GSTT1* genotype, with high enzyme activity, are better protected against ethylene oxide and other alkylating agents (Haufroid et al., 2007). Among controls, we did not observe an influence of *GSTT1* gene polymorphism on the frequencies of SCEs and CAs. However, it should be emphasized that the control group includes individuals occupationally not exposed to xenobiotics. For this reason, we do not expect particularly high values of SCEs and CAs, and thus it is difficult to observe an effect exerted by certain genetic polymorphisms on the levels of these cytogenetic biomarkers.

In conclusion, our results suggest that a continuous long-term exposure to low doses of anaesthetic gases could result in increased levels of SCEs among exposed. These data (results) emphasize the need to develop safety programs and the importance of the health surveillance of workers occupationally exposed to air pollutants, such as anaesthetic gases. In this scenario, the implementation of security measures in this sector, as well as good practice campaign,s may be crucial to decrease this professional health risk.

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TABLE 1. Demographic Characteristics of the Studied Groups

Characteristic	*Anesthetist*	*Control*
Subject	21	21
Sex		
Males	15	13
Females	6	8
Age (years)		
Mean ± SD	35.524 ± 4.332	35.857 ± 7.059
Range	28–46	22–48
Years of employment		
Mean ± SD	8.619 ± 4.364	8.190 ± 4.686
Range	2–17	1–20
GSTT1 genotype		
GSTT1 positive	15	14
GSTT1 null	6	7

SD, standard deviation.

TABLE 2. Frequency of SCEs and RI Values in Metaphases of Lymphocytes from Exposed and Controls

Group	*N*	*NSM*	*SCEs*	*SCEs/NSM ± SE*	M_1	M_2	M_3	*RI ± SE*
Anesthetist	21	1050	7528	7.170 ± 0.356[a]	989	766	301	1.630 ± 0.069[b]
HFIs	12	600	4989	8.315 ± 0.282[c]	621	426	109	1.500 ± 0.089
Non-HFIs	9	450	2539	5.642 ± 0.286[c]	368	340	192	1.804 ± 0.083
Males	15	750	5226	6.968 ± 0.382	722	539	206	1.612 ± 0.089
Females	6	300	2302	7.673 ± 0.822	267	227	95	1.677 ± 0.102
GSTT1 positive	15	750	5134	6.845 ± 0.389[d]	737	534	230	1.663 ± 0.076
GSTT1 null	6	300	2394	7.980 ± 0.727[d]	252	232	71	1.548 ± 0.158
Control	21	1050	5149	4.904 ± 0.368[a]	727	837	535	1.908 ± 0.042[b]
HFIs	7	350	2440	6.971 ± 0.198[e]	235	301	164	1.899 ± 0.044
Non-HFIs	14	700	2709	3.870 ± 0.310[e]	492	536	371	1.899 ± 0.044
Males	13	650	3105	4.777 ± 0.502	459	539	301	1.877 ± 0.045
Females	8	400	2044	5.110 ± 0.657	268	298	234	1.958 ± 0.075
GSTT1 positive	14	700	3391	4.844 ± 0.473	446	572	382	1.954 ± 0.048
GSTT1 null	7	350	1758	5.023 ± 0.741	281	265	153	1.814 ± 0.061

[a] $P = 0.001$; [b] $P = 0.005$; [c] $P = 0.008$; [d] $P = 0.046$; [e] $P = 0.018$.
N, number of analysed subjects; NSM, number of scored metaphases; SCEs, sister chromatid exchanges; SE, standard error; RI, replication index.

TABLE 3. Frequencies of Chromosomal Aberrations in Lymphocytes of Exposed and Control Subjects

Group	*N*	*NSM*	*B′*	*B″*	*Dic*	*AF*	*Total CAs*	*Total CAB*	*CAs/NSM Mean ± SE*	*CAB/NSM Mean ± SE*
Anesthetist	21	4200	44	21	5	6	76	73	0.018 ± 0.002	0.017 ± 0.002
Males	15	3000	38	16	4	5	63	60	0.021 ± 0.003	0.020 ± 0.002
Females	6	1200	6	5	1	1	13	13	0.011 ± 0.002	0.011 ± 0.003
GSTT1 positive	15	3000	32	13	3	3	51	48	0.017 ± 0.002	0.016 ± 0.002
GSTT1 null	6	1200	12	8	2	3	25	25	0.021 ± 0.005	0.021 ± 0.005
Control	21	4200	35	8	6	15	64	61	0.015 ± 0.002	0.015 ± 0.002
Males	13	2600	17	4	5	11	37	36	0.014 ± 0.002	0.014 ± 0.002
Females	8	1600	18	4	1	4	27	25	0.017 ± 0.006	0.016 ± 0.006
GSTT1 positive	14	2800	19	5	5	11	40	40	0.014 ± 0.003	0.016 ± 0.004
GSTT1 null	7	1400	16	3	1	4	24	23	0.017 ± 0.004	0.016 ± 0.004

N, number of analyzed subjects; NMS, number of scored metaphases; CAs, chromosome aberrations; CAB, cells with aberrations; B′, chromatid breaks; B″, chromosome breaks; Dic, dicentric chromosome; AF, acentric fragments; inv, inversions; Rad, Tri or tetraradials; SE, standard error.

TABLE 4. Multiple Regression Analysis of Confounding Factors on SCEs and CAs Frequencies in Lymphocytes of the Study Groups

	SCEs Frequency			*CAs Frequency*		
			95% CI			*95% CI*
CF	*β-co*	*P-Value*	*Lower–Upper*	*β-co*	*P-Value*	*Lower–Upper*
Anesthetist						
Age	10.473	0.365	–13.214–34.160	–0.249	0.381	–0.803–0.322
Y.E.	–8.443	0.460	–31.951–15.066	–0.331	0.229	–0.228–0.889
Control						
Age	1.967	0.702	–8.663–12.598	0.136	0.271	–0.116–0.388
Y.E.	0.940	0.903	–15.073–16.952	–0.029	0.875	–0.408–0.351

CF, confounding factor; *β*-co, *β*-coefficient; Y.E., years of employment.