A PRELIMINARY STUDY OF SISTER CHROMATID EXCHANGE IN PERIPHERAL BLOOD LYMPHOCYTES OF SPANISH ADULT SMOKERS COMPARED TO NON-SMOKERS

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ABSTRACT

According to the International Agency for Research on Cancer tobacco smoke is uniquely important as a cause of human cancer and, it has caused about half of all cancer deaths in men and increasing number of deaths in women in some groups of people. Because of this worldwide health problem, the aim of this study was to estimate, for the first time, the Sister Chromatid Exchanges (SCE) frequency and the HFC parameter on the Spanish population, grouped in light and heavy smokers and non-smokers in order to evaluate tobacco genotoxicity. Moreover we described the SCE pattern with a new parameter. A local sample of 101 adults of smokers (n=48) and non-smokers (n=53), aged from 18 to 49 years, was studied using SCE levels in peripheral lymphocytes. The habit of heavy smoking (≥20 cigarettes per day) increased significantly the SCE frequency and the HFC parameter. Neither age nor sex did significantly influence the frequencies in our studied group. Our findings help the initiative of extending smoke-free legislation in the direction of the new smoking law, in place since 2010 in our country.

1. Introduction

Tobacco smoking is a worldwide habit and one of the major health problems in the world. In 2009, according to the International Agency for Research in Cancer (IARC), this number increased up to more than 1 billion people [1]. Tobacco is a natural product that consists of more than 3800 constituents (4800 compounds in tobacco smoke) ranging from small molecules such as hydrocarbons, terpenes, carbonilics, phenols, or nitriles, to macromolecules as cellulose, lignin or nucleic acids. These compounds suffer transformations due to the high temperatures achieved during the tobacco consumption and some of them are toxics and carcinogenics [2]. Among these compounds more than 50 are known carcinogens, such as polycyclic aromatic hydrocarbon (PAHs), N-nitrosamines, aromatic amines, and trace metals [3]. Actually, it was stated that tobacco smoking is the single largest cause of cancer worldwide [1]. If we take into account the data, 5–6 million deaths each year are attributed to tobacco use and this annual toll may increase to 10 million within the next 20–30 years [3]. Concerning its carcinogenicity, in the special report from the IARC it has been compiled those tumour sites for which the evidence of causality is sufficient (oral cavity, oropharynx, nasopharynx, and hypopharynx, oesophagus (adenocarcinoma and squamous-cell carcinoma), stomach, colorectum, liver, pancreas, nasal cavity and paranasal sinuses, larynx, lung, uterine cervix, ovary (mucinous), urinary bladder, kidney (body and pelvis), ureter, bone marrow (myeloid leukaemia) [1].

In the last decades, the genotoxicity of cigarrette smoking has been assessed by several assays. Our study was thought to assess the impact of cigarette smoking on the Spanish population, therefore we follow a Prevention Technical Note emitted by the Spanish Ministry of Labour and Social Affairs so-called "Biological control of exposure to genotoxic agents: cytogenetic techniques" [4]. According to this Technical Note the

exposure to tobacco smoking can be evaluated with two different cytogenetic techniques, chromosomal aberrations and Sister Chromatid Exchanges (SCE). However, it is said that faced with certain chemical agents, induction of SCE may have a greater sensitivity to chromosomal aberrations, and may include genotoxic effects at concentrations up to 100 times lower than those required to produce chromosomal aberrations [4]. Several studies have indicated a potential relationship between SCE and other indicators of genomic instability, such as fragile sites and susceptible sites to ionizing radiation [5]. Particularly, SCE occur when there is a symmetrical exchange of DNA segments between two sister chromatids of a duplicating chromosome being formed during the S phase of the cell cycle and can be induced by chemicals that are S phase-dependent DNA-damaging agents [6].

The literature demonstrated the importance of using SCE technique to evaluate the tobacco-related genotoxic effect [3, 7-9]. The analysis of SCE is frequently complemented with the analysis of cells containing a high frequency of SCE (HFC) which are defined as cells whose SCE frequency exceeds the 95th percentile of the SCE distribution in a pooled data set from control individuals [10]. This HFC measure is often more sensitive for the evaluation of the exposure to genotoxic agents, thus it increases the sensitivity of the SCE assay when the effect of an exposure is not obviously detected by differences in mean SCE values [9]. Some authors have postulated that HFCs could represent a subpopulation of DNA repair-deficient lymphocytes. Others however stated that HFCs may represent a subpopulation of longer lived lymphocytes which have an increased likelihood of accumulating a larger number of lesions during the Go phase *in vivo*, thus showing a predisposition to SCE. In the latter case HFCs would be more properly used for evaluation of exposure to genotoxic agents [10]. Moreover we analyzed the SCE distribution patterns in metaphases which

allowed checking the uniform or non-uniform distribution of SCE among the chromosomes in each metaphase.

In Spain, a smoking law, in place since January 1st, 2006, restricted smoking at the workplace and in public places, including recreation venues (bars and restaurants) [11], but smoking was not completely prohibited in bars and restaurants [12]. Later, in 2010, the Spanish Government modified this previous legislation in order to reduce smoking in public places and to eliminate the medium-and long-term smoking [13].

Hence, the aim of this work, as a preliminary study, was to assess the tobacco-related genotoxic effect in peripheral blood lymphocytes on the Spanish local population.

2. Materials and methods

2.1. Participants

A sampling frame of one hundred and one adults (55 females and 46 males) aged 18-49 years were sourced from an existing internal local panel supplied by the local sanitary authorities. The group of control group (non-smokers) included 48 subjects and the smoking group 53. The survey was conducted from December 2003 to November 2004 and approved by the Ethical Committee of Hospital La Fe (Valencia, Spain).

2.2. Culture conditions and stain technique

Human peripheral blood samples were collected in sterile vacutainer tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) containing lithium heparin as anticoagulant, after their informed consent. For each treatment, separate cultures were set up by mixing 0.75 mL of whole blood with 5 mL of PB-MaxTM Karyotiping medium (Gibco, Barcelona, Spain) and incubated 72 hours at 37 ºC. 150 μL of Colcemid® (Gibco, Barcelona, Spain) from a stock solution of 10 μg mL^{-1} were added 2 hours before harvesting to stop the cell culture in metaphase. Carnoy's

lymphocytes fixative solution was prepared with methanol (Merck, Ramstadt, Germany) and acetic acid (Panreac, Barcelona, Spain). Cytogenetic analyses were carried out by using a conventional microscope (Izasa, Barcelona, Spain) and an image analysis system with the IKAROS-software (MetaSystems).

Two- to three-day-old slides were stained with Fluorescence plus Giemsa stain technique. Briefly, old slides were treated 20 min in Hoechst 33258 at room temperature. The slides were washed with distillated water. The slides were covered with 2xSSC and treated with UV (300 W) for 2 min. Once washed with water and dried 30 min, the slides were stained with Leishman (Merck, Ramstadt, Germany) for 5 min being useful in the identification of first-, second- and third-division metaphases (Fig. 1).

2.3. Cytogenetic analysis

The incidence of SCE, HFC and SCE distribution patterns (Fig. 2) was determined from the analysis of 50 second division metaphases for each individual. The evaluation of SCE scores in lymphocytes included scoring total exchanges in total number of analyzed cells for each treatment to establish its frequency (Y_{SCE}) in 46 chromosomes per cell.

2.4. Statistical analysis

For each cell, its SCE frequency was calculated. Alternatively, we also computed the number of HFCs per individual according to the method proposed by Carrano and Moore [14]: a HFC cells was defined as a cell which exceeds the 95th percentile of the SCE distribution from control individuals. As a measure of differences in SCE distribution patterns, we estimated for each cell the number of SCEs per affected chromosome. A higher $\frac{n^{o}\ of\ SCE}{n^{o}\ affected\ chromosomes}$ ratio representing a more clustered SCE pattern. . Since the estimated ratio for a random distribution of SCEs in a cell grows as a

function of the number of SCEs (Fig. 3), we subtracted the expected ratio from our observed ratio as a measure of deviation from a random distribution pattern.

According to Laugesen and Swinburn [15], subjects were categorized into three categories: light smokers (who smoke less than 20 cigarettes per day), heavy smokers (defined as those who smoke 20 or more cigarettes per day) and non-smokers.

SCE frequencies and ratio distribution patterns were modeled in terms of age, sex and smoking status using a linear mixed model approach. This models regarded individuals as a random factor to correct for intra-individual correlation among cells. Counts of HFCs per individual were modeled with the same explicative variables using a negative binomial distribution. The negative binomial distribution was preferred over the poisson due to the presence of overdispersion in the data.

All interactions between variables were assessed (age-sex, age-smoking, sex-smoking). After fitting of the saturated models with all the variables and its interactions, a model selection procedure was performed using second-order Akaike Information Criterion (AICc) to find the best model. All statistical analyses were carried out using the R software (version 2.15.2).

3. Results

3.1. SCE analysis

SCE frequency had a mean of 8.19 and a 95% CI from 7.88 to 8.5. The best model, according to the model selection procedure, included only the variable smoking, rejecting the effect of age and sex (Table 1).

The effect of smoking, with a p-value < 0.001, was statistically highly significant. Both, heavy smoking and light smoking status were associated with a significant increase in SCE frequency (Fig. 4). According to the model estimates for the coefficients, the mean frequency of SCE in non-smokers cells was 7.46 (95% CI [7.12;

7.81]), in the light smoking group it was 1.21 (95% CI [0.6; 1.88]) units higher and in the heavy smoking group the mean was 2.02 (95% CI [1.27; 2.78]) units higher.

3.2. HFC Analysis

The results of our second analysis, regarding count of HFCs per individual, were similar. The distribution of SCEs had a 95th percentile of 16 (Fig. 5). Mean number of HFCs/individual was 3 with a 95% CI [2.35; 3.85]. The best model, according to the model selection procedure, included only the variable smoking, rejecting the effect of age and sex (Table 2).

The effect of smoking, with a p-value < 0.001, was statistically highly significant. Both, heavy smoking and light smoking status were associated with a significant increase in HFC count per individual. According to the model estimates for the coefficients, the mean HFC count per individual in the non-smoking group was 1.38 (95% CI [0.99; 1.93]), in the light smoking group it was 4.07 (95% CI [2.5; 6.69]) and in the heavy smoking group the mean was 5.83 (95% CI [3.38; 10.36]) (Fig. 6).

3.3. SCE distribution pattern

Results of our analysis regarding SCE distribution pattern agree with the other two. The best model included only the variable “smoking status” (p-value = 0.029). (Table 3). Both, heavy and light smoking status, were associated with a significant increase in the ratio.

DISCUSSION

This is the first study, to our knowledge, to assess the tobacco-related genotoxic effect in peripheral blood lymphocytes by SCE on the Spanish population. It is known that SCE is one of the most extensively used biomarkers to assess the genotoxic potential of mutagenic and carcinogenic agents because its expression reflects possible alterations of the cell cycle or genetic damaging events at the chromosomal level [16].

Although we thought to include in the statistical model the age and sex variables, the analysis showed that both variables did not have influence in the results; therefore they were not included in the final model. Our results match those of Husum et al. [17] who reflected that, in their study, sex did not significantly influence the SCE frequency. However, opposite results have been also observed; Anderson et al. observed that females had significantly higher SCE frequencies than males, and cigarette smoking significantly increased such values after correction for sex [18]. Lazutka et al. [7] reported similar results, indicating that SCE increased with age and cigarette smoking intensity, and higher SCE frequencies were observed in females. Therefore, sex and age are variables that would be important to include in the study to evaluate their influences in the results.

Concerning the results of SCE frequency with only the variable smoking or not smoking, some studies show similar results to ours. Lambert et al. [19] reported that, among 43 subjects, those who were cigarette smokers had significantly higher SCE frequencies than non-smokers. They observed a stepwise increase of about 15% in the average SCE-frequency among moderate (< 10 cigarettes per day) and heavy smokers (≥10 cigarettes per day) and they ascribed their results to benzo(a)pyrene (BP). These results were contradicted by Hopkin et al. [20], who evidenced that mainstream smoke cigarettes is a potent inducer of SCE whose results were confirmed twenty years later by De Marini et al. [21]. In another study, Wulf et al. [22] found that the SCE mean values in three categories of cigarette smokers (high-tar with filter, high-tar without filter and low-tar with filter) were statistically higher than the mean value observed in the non smokers group; moreover, they also detected that even the SCE mean value for pipe and cheroot smokers were always higher than the non smokers group. Once more, an increase in the incidence of SCEs in the peripheral lymphocytes of smoker volunteers

(6.5 +/- 0.3) compared to non-smokers (4.1 +/- 0.2) was observed by Sardaş et al. [23] where both the duration of smoking and the number of cigarettes smoked per day appeared to influence SCE frequency; specifically, those who smoked more than 10 cigarettes per day and those who had habitually smoked for over 10 years had higher SCE frequency. Ben Salah et al. [9] also indicated that the mean frequency of SCE per cell was significantly higher in smokers than in non smokers. Another recent study, the group also evaluated the genotoxicity of cigarette smoking in volunteers from Jordan using the SCE assay [4]. Our results were in agreement with them since SCE frequencies in the cigarettes smoking group significantly increased compared with those of non smokers. Furthermore they pretended to compare SCE value between cigarette and waterpipe smokers and found that SCE frequencies in the waterpipe smoking group were higher than in control group (non smokers) and even higher than in cigarette smoking group.

In an attempt to carry out a more sensitive and accurate analysis, we analyzed the HFC parameter since HFCs may represent a more sensitive criterion for assessment of exposure, such as smoking habit, than mean SCE values [9]. With the analysis of HFC we found that again, heavy smoking and light smoking status were associated with a significant ($p<0.0001$) increase in HFC count per individual. Some authors have postulated that HFCs could represent a subpopulation of DNA repair-deficient lymphocytes. Interpreting SCE as a signal of DNA damage and due to the SCE frequency decreases through repeated cell cycles as a consequence of DNA damage removal, it can be inferred that HFCs, although initially more damaged, succeeded in removing most SCE inducing lesions over three cell cycles, above all after the first cycle. Therefore, the greater number of SCEs observed in HFCs could be attributable to a higher level of initial damage and not to repair deficiencies. In the present study by

analyzing HFCs we also consider those lymphocytes that could have been accumulating lesions from tobacco compounds.

From the analysis of the SCE distribution pattern, both heavy and light smoking status, were associated with a significant increase in the ratio. This means that the SCE in not uniformly distributed among the 46 chromosomes in the cell, it seems that there is a tendency in accumulating the SCE only in one chromosome rather than being distributed among all of them. The mechanisms by which some compounds can induce SCE are different; some polyphenols damage the DNA and may produce SCE by arresting S phase through cleaving the DNA [24], others, such as antitumor antibiotics (i.e., mytomycin), damage DNA in the presence of a redox-active metal ion such as Fe^{2+} or Cu^{+}, as well as molecular oxygen [25]. Considering this last mechanism, it can be thought that among the wide variety of tobacco compounds some of them can act like the antitumor antibiotics, this means that they would suffer activation by a redox complex with metal ions from DNA and this active products, usually with a short life, would produce a clustering damage in the same chromosome. In our analysis the fact that one chromosome is more damaged that another one could not be attributed to one specific compound due to the complex tobacco composition. However, it has been described that some molecules such as nitroimidazole derivates not affect all the chromosomes equally supporting the idea of the "specific genomic targets". Even analyzing SCE frequencies by chromosome, these authors observed that certain chromosomes showed higher susceptibility to that treatments within the q arm of a specific chromosome and moreover, they specified which region inside the band was more affected and emphasized the importance of the so-called "hot spots" and the validity of SCE biomarker as cytogenetic indicators of genomic fragility [5, 16].

It is worth mentioning that the global concern about the tobacco consuming health

effects has a key date in 1969, when the International Agency for Research on Cancer (IARC) initiated a programme for the evaluation of the carcinogenic risk of chemicals to humans involving the production of critically evaluated monographs on individual chemicals [26]. Thanks to this, in the last years, evidence has been accumulated regarding that carcinogenicity of tobacco smoke. *In vitro* toxicology studies of tobacco and tobacco smoke have been a useful tool to understand why tobacco use causes cancer and a plethora of *in vitro* assays are available to assess tobacco smoke modes of action, mostly using non–human cell models. SCE is one assay considered for this assessment, using mainly cultured CHO (Chinese Hamster Ovary) cells or human lymphocytes, in part because it is a technical challenge to obtain a high percentage of synchronized metaphases using cell types. Johnson et al. reviewed the literature available for this assays and observed that Cigarette Smoke Condesates (CSCs) and Total Particulate Matter (TPM) induce SCEs in *in vitro* studies in a concentration-dependent manner [6]. This means, that they CSCs and/or TPM should have genotoxics agents which can induce SCE. In one Spanish study among 10 tobacco brands, the authors carried out an extensive analysis to elucidate the main compounds found in the mainstream tobacco smoke. The analysis of the vapor fraction showed that the major compounds were by far CO and CO_2 and it is well known that CO is a poison. Besides them, the small aldehyde molecules present in tobacco smoke are particularly harmful specially the acetaldehyde. Other remarkable compound found was the 1,3-butadiene which presents the highest cancer risk index of all constituents of cigarettes smoke [2] and which metabolite 1,2:3,4-diepoxybutane was shown to induce SCE *in vitro* in human lymphocytes [27]. Furthermore, 85 components were identified in the particulate matter such as nicotine, polycyclic aromatic compounds or nitrosamines which are known as carcinogens [26].

In vitro studies are correlated with those *in vivo* studies mentioned herein and with

our results. Unfortunately the *in vitro* toxicology methods provide data that cannot reliably be extrapolated to infer human cancer risk and were intended primarily as screening methods for chemicals to identify possible human carcinogen [6]. Thus, *in vivo* studies are necessary to detect and evaluate the impact of tobacco habit in human health with the use of biomarkers.

From our study, it can be concluded that the frequencies of SCE and HFC from the cigarette smoking group significantly increased compared with those of non-smokers. Moreover it seems that there exists a distribution pattern that concentrate the damage in some chromosomes instead of a distributing it uniformly which can be interpreted in some kind of genomic susceptibility to tobacco compounds. Our findings are in agreement with other authors when using the SCE assay to confirm the smoking induced chromosome damage and justify the need to launch campaigns and laws to reduce the cigarette consumption.

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Figure caption

Fig. 1. Image illustrating 3 consecutive divisions of a lymphocyte after Fluorescence plus Giemsa staining. In the image it can be observed a) lymphocytes in first division, b) lymphocyte in second division (SCE counting) and c) lymphocyte in third division.

Fig. 2. Metaphase in second division showing chromosomes containing different number of SCEs: a) 1 SCE, b) 2 SCE, c) 3 SCE. The ratio is calculated according to this formula: Ratio = $a \cdot 1 + b \cdot 2 + c \cdot 3$ (total SCEs)/$a + b + c$ (affected chromosomes). For this metaphase: Ratio = 29/20 = 1.45. Adjusted ratio = 1.45 − expected ratio for 29 SCE (1.35) = 0.1

Fig. 3. Estimated ratio for a random distribution of SCEs in a cell.

Fig. 4. Comparison of SCE frequencies distribution between non-smokers, light and heavy smoker cells and towards the population mean.Distribution of the mean frequency of SCE in the population.

Fig. 5. Distribution of SCEs with a 95th percentile of 16.

Fig. 6. Comparison of HFCs count per individual between non-smokers, light and heavy smokers and towards the population mean.