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Cell proliferation kinetics and genotoxicity in lymphocytes of smokers living in mexico city

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Genotoxicity caused by tobacco smoke was assessed in peripheral blood lymphocytes of smokers living in Mexico City by determining sister chromatid exchange (SCE), cell proliferation kinetics (CPK), replication index (RI) and mitotic index (MI). Nicotine levels, and its major metabolite cotinine, were also estimated in urine samples using gas-chromatography-mass spectrometry to quantify smoking intensity. The outcome of the analysis and the comparison of the 77-smoker group with a non-smoking control group showed that moderate and heavy smokers exhibited significant differences ($P < 0.001$ and $P < 0.05$, respectively) in CPK, with an underlying delay in the cellular cycle; similarly, RI was significantly different in these groups ($P < 0.001$ and $P < 0.0001$, respectively). There were significant correlations ($P < 0.05$) between age and number of years the subject had been smoking, as well as between RI and nicotine and cotinine levels and between CPK (M1, M2 and M3) and nicotine and cotinine levels. Smokers were classified for the analysis according to the nicotine levels (it is in relation to number of cigarettes smoked per day) found in urine (ng/mL) as: light (10–250), moderate (251–850) and heavy (851–4110). Significant differences in CPK were found ($P < 0.05$) between moderate and heavy smokers and non-smokers. Significant differences in RI were found between moderate ($P < 0.001$) and heavy smokers ($P < 0.0001$) and non-smokers, but not for the light smoking group. MI was determined in 57 of the smokers, whereas SCE frequency was only recorded in 34 smokers. Both parameters yielded no significant differences, nor correlations with any of the assessed variables. In conclusion, cytokinetic and cytostatic effects were mainly detected in heavy and moderate smokers. Cell cycle delay and RI decrease were found in all 'healthy' smokers. The nicotine and cotinine exposure (causing oxidative damage to DNA) may have implications in the decrease in cell replication due to direct damage to DNA and/or a decrease in the DNA repair mechanisms. Alternatively, nicotine and cotinine may possibly induce apoptosis. *Human & Experimental Toxicology* (2007) **26,** *715–722*

Key words: cell proliferation kinetics; cotinine; cytostatic effect; cytotoxicity; genotoxicity; human lymphocytes; mitotic index; nicotine; replication index; sister chromatid exchange; smokers

Introduction

Tobacco smoke contains both particulate and gaseous components; approximately 60 chemical compounds identified in tobacco smoke are considered carcinogenic in animals and 10 in humans.[1]

Cigarette smoking has been associated with a number of medical conditions such as bronchitis, emphysema, lung cancer, cancer in other organs, mood (depression) and sleep disorders, as well as causing an increase in mortality.[2,3]

According to The World Bank (1999)[4] tobacco smoke is responsible for the deaths of almost five million people each year worldwide. Between 1990 and 1995 cigarette consumption in the USA and Canada decreased, but in Central and South America there was an increase in demand however, consumption levels did not reach those of developed countries.[5] In Mexico, the number of smokers increased from 9.2 million in 1988 to 14.3 millions in 1998. It is estimated that more than 6000 deaths are caused annually by lung cancer and more than 12 000 by heart disease derived directly from smoking.[6,7]

Genotoxicity caused by tobacco smoke has been a matter of controversy. For example, some authors

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have reported a smoke-related increase in the frequency of sister chromatid exchanges (SCE),[8–13] while others found no such increase.[14–16] Several studies have shown an association between certain factors or lifestyles and smoking habit. Soper *et al.*[17] found the induction of SCE increased with the age of the smoker, while other authors found no relationship between age and genotoxicity.[18–20] However, an increase in SCE levels has been reported in subjects older than 60 years compared to newly born or children.[21–23] Similarly, variations in replication index (RI) values due to smoking have been reported, reflecting inhibition in DNA synthesis.[24,25] Those studies also showed that cellular proliferation kinetics (CPK) was inversely correlated to the age of the smoker. Other studies have related the effects of smoking to the history of individuals as smokers, that is, dose-related exposure history, measured in cumulative pack-years and have shown a high SCE frequency not only in heavy smokers but also in those with a long history of smoking.[10,25] In addition, other characteristics have been evaluated in relation to tobacco smoking such as the gender of smoker[26] and coffee and alcohol consumption.[27] Barale *et al.*,[26] measured SCE frequency and micronuclei (MN) in lymphocytes of 1650 subjects, of which 427 were smokers, these authors found that the number of cigarettes smoked per day raised the SCE frequency significantly. This increase has also been reported to be greater for women smokers and older subjects compared to that for men and young people. Mean values of SCEs have also been found to be lower in children and are known to increase with age.[26,28–31] However, Sarto *et al.*[32] found no relationship between SCE frequency and age and/or gender of smokers.

One of the principal components of tobacco smoke is nicotine.[33] Cotinine, its main metabolite, has an average half-life in the body of 15–17 h compared with 2.6 h for nicotine. Both compounds are currently used as exposure biomarkers.[34,35]

"Nicotine is the major pharmacologically active substance in cigarette smoke and plays an important etiological role in the development of lung cancer. Incidence of cancer may be related to oxidative damage to host genome by nicotine."[36] Approximately 50% of long-term cigarette smokers die prematurely from the adverse effects of smoking, including lung cancer and other illnesses.[37]

The genotoxic effects of tobacco smoking have been mainly assessed by studying the variation in SCE frequency in lymphocytes from peripheral blood of smokers. However, studies on the effects of smoking on populations have not always included the assessment of DNA damage obtained by the analysis of CPK, RI and mitotic index (MI) and effects of occupational or environmental conditions on exposed or non-exposed subjects. This study reports the genotoxic and cytotoxic effects on smokers determined by SCE, CPK, RI and MI in peripheral blood lymphocytes. In addition, we report levels of nicotine and its major metabolite, cotinine, in the urine of smokers as biomarkers of smoke exposure.

Materials and methods

The smokers that participated in this study were recruited from the Anti-Tobacco Clinic in the Instituto Nacional de Enfermedades Respiratorias (National Institute of Respiratory Diseases) in Mexico City. Blood samples were collected while the participants were still smokers; each volunteer was seeking treatment for giving up smoking. Seventy-seven smokers and 77 non-smokers were recruited for the study.

The classification groups for this study were defined, according to the concentration of nicotine in urine, as: light smokers (10–250 ng/mL), moderate smokers (251–850 ng/mL) and heavy smokers (851–4110 ng/mL). The control group consisted of non-smokers that were not exposed to tobacco smoke at least in their homes or workplaces. The age and gender composition of the control group was similar to that of the smoking group.[38] In addition, it was verified that none of the participants were on medication, alcoholics or regularly exposed to genotoxic substances.

Human lymphocyte cultures

Venous blood samples from 77 smokers and 77 non-smokers were taken with heparinized vacutainer tubes and transferred to the laboratory within a few hours of taking the sample. Blood (400 μL) from each sample was added to 4.5 mL RPMI medium 1640 with L-glutamine (Gibco) plus 0.2 mL phytohemagglutinin (Gibco). The cultures were incubated at 37°C for 72 h. After 24 h, 5-bromodeoxyuridine (5-BrdU, Sigma) was added to the culture medium to get a final concentration of 5 mg/mL. Seventy-one hours after starting the culture, 4 mg/mL of colchicine (Sigma) were added and 1 h later the cells were harvested by centrifugation, given a hypotonic shock (0.075 M KCl) and fixed in a methanol-acetic acid mixture (3:1). Slides were made by placing a drop of cell suspension onto the slide and allowing this to air dry. The cells were later stained in Hoechst 33258 solution (5 mg/mL) for 20 min in the dark, mounted with tap water, irradiated for 3–4 h with UV light and finally placed in Giemsa-distilled water (1:50) for 30 min.

The RI enables the evaluation of cytotoxic agents that affect cell proliferation kinetics (CPK) and that can be determined by the alteration of the frequencies of the metaphases in the first (M1), second (M2) and third (M3) divisions, analyzing 100 consecutive mitoses per individual. The RI was calculated as follows: RI = (1M1 + 2M2 + 3M3)/100.[39,40] The mitotic index evaluates the cytotoxicity of chemical agents.[41] It was determined by scoring the number of dividing cells in 3000 cells, for each donor. Cytogenetic changes for each subject were evaluated by assessing the number of SCEs in 50 second-division metaphases with at least 40 chromosomes.[19] In order to avoid bias, slides were marked with a code to keep the origin unknown (smoker/non-smoker).

Detection and quantification of nicotine and its metabolite cotinine in urine samples from smokers

Nicotine and cotinine in urine was quantified using gas chromatography-mass spectrometry. The method of Hutchinson *et al.*,[42] was followed, taking into account their respective deuterated internal standards. Analyses were isolated by liquid–liquid extraction coupled to centrifugation and evaporation. The sensitivity test gave 10 ng/mL for nicotine and 100 ng/mL for cotinine. Calibration curves were within ranges 1–3000 ng/mL and 1–10 000 ng/mL for nicotine and cotinine, respectively. All data were corrected for recovery efficiencies.

Statistical analysis

The chi-squared (X^2) test was used for mitotic index, as well as for comparison of M1, M2 and M3 values. Student's *t*-test was applied to RI and SCE results. Correlations among different variables were established using Spearman's rank order correlations test.

Results

Human lymphocyte cultures of 77 smokers and 77 non-smokers matched by age and gender were assessed to determine the effects caused by tobacco smoke on RI, MI and SCE.

Table 1 shows number of cigarettes smoked per day, smoking time (years the subjects have smoked), age, nicotine and cotinine levels in urine (ng/mL), CPK, RI of 61 smokers; the rest of this group did not reach the 100 required metaphases to estimate RI. Parts of the decomposed Chi-squared test were used to compare significant differences ($P < 0.05$) between M1, M2 and

Table 1 Results from 61 individuals grouped into three smoking categories: light (L/S), moderate (M/S) and heavy (H/S). Cigarettes per day; years the subjects have smoked; age; nicotine and cotinine concentration in urine; effect on CPKs reflected in the proportion of first (M1), second (M2) and third (M3) metaphase stages and RI

Individual	*C/Day*[a]	*T/S*[b]	*Age*	*Nicotine (ng/mL)*	*Cotine (ng/mL)*	*Smoker CPKs*				*Non-smoker CPKs*			
						M1	*M2*	*M3*	*RI*	*M1*	*M2*	*M3*	*RI*
L/S													
Mean	19.0	34.4[c]	51.2	117.8[d]	950.1	51.1	31.3	17.6	1.67	32.5	42.5	25.0	1.93
SE	3.88	3.16	3.03	31.5	212.1	8.54	5.3	3.70	0.11	4.81	3.0	2.8	0.07
M/S													
Mean	21.7	30.9[c]	51.2	494.0[d,e]	1073.7[f]	56.3*	28.7*	15.0*	1.60**	31.4	41.0	27.6	1.97
SE	2.33	3.58	3.1	44.8	152.9	5.0	4.0	2.6	0.07	2.08	1.65	2.141	0.03
H/S													
Mean	26.1	27.7[c]	44.8	1688.6	1820.3[f,g]	55.0*	28.0*	17.0*	1.63**	34.1	41.5	24.3	1.90
SE	2.12	1.97	2.20	152.5	193.1	3.7	2.70	1.92	0.05	1.87	1.26	1.32	0.03

[a]Cigarette/day
[b]Years subjects have smoked
X^2 test was applied to compare M1, M2 and M3 values
M/S *Significance, $P < 0.002$
H/S *Significance, $P < 0.05$
Student's t-test was applied in RI
M/S **Significance, $P < 0.001$
H/S **Significance, $P < 0.0001$
Spearman's rank order correlations test was applied among variables:
Significance, $P < 0.05$
[c]L/S, M/S and H/S, smoking time and age
[d]L/S, M/S, nicotine and CPK (M1, M2, M3)
[e]M/S, nicotine and RI
[f]M/S and H/S, cotinine and RI from smokers
[g]H/S, cotinine and CPK (M1, M2, M3)

M3 values of smokers with the respective controls (e.g., an average of 56.3 first division metaphases was counted for moderate smokers and 55.0 for heavy smokers compared to 31.4 and 34.1 respectively, for non-smokers). Likewise, RI was significantly different for both moderate ($P < 0.001$) and heavy smokers ($P < 0.0001$) when Student's *t*-test was performed. The variables considered in this study were correlated among themselves by applying Spearman's rank order correlations test: As a result, in the three smoker groups studied significant differences ($P < 0.05$) were obtained between time and age of smokers; in light and moderate smokers nicotine levels in urine was significantly correlated with CPK (M1 M2 and M3), whereas for moderate smokers, a significant correlation was found between nicotine and RI. Likewise, cotinine was significantly correlated with RI in moderate and heavy smokers. Finally, cotinine and CPK were also correlated in heavy smokers.

The 61 smokers shown in Table 1 were classified according to their nicotine levels in urine (ng/mL) as: light (10–250), moderate (251–850) and heavy (851–4110). Significant differences in CPK were found ($P < 0.05$) between moderate and heavy smokers and non-smokers. Significant differences in RI were found between smokers and non-smokers for moderate ($P < 0.001$) and heavy smokers ($P < 0.0001$), but not for the light smoking group.

Nicotine levels in urine of light smokers fluctuated between 10 and 248 ng/mL, with an average of 117.8. In moderate smokers these values ranged between 290 and 810 ng/mL, with an average of 494.0; and in heavy smokers the values were between 861 and 4102 ng/mL, with an average of 1688.6. Average cotinine levels for the heavy, moderate and light groups were: 1820.3 ng/mL (range from 965 to 4946); 1073.7 ng/mL (range from 101 to 2364) and 950.1 ng/mL (range from <100 to 1814), respectively.

Of the 77 smokers evaluated, 16 were not assessed since the 100 mitoses required for obtaining RI were not reached. Moreover, MI was determined in only 57 smokers as not all the samples exhibited 3000 cells in the interphase stage. No significant differences in MI were found between smokers and controls (Table 2).

Tobacco smoke genotoxicity was assessed by SCE analysis. SCE frequency could not be measured for 43 of the smokers because the required 50 second-division metaphases with more than 40 chromosomes were not observed. No significant difference in SCE frequency was found between non-smokers and the 34 smokers in which SCE was determined (Table 3).

Table 2 Results from 57 individuals classified into one of the three groups of smokers: light, moderate and heavy. Cigarettes per day; years the subjects have smoked; age and mitotic index from smokers and non-smokers

Individual	*C/day*	*T/S*	*Age*	*Smokers Mitotic index*	*Non-smokers Mitotic index*
Mean	23.6	29.7	46.9	5.2	4.3
SE	12.0	12.7	12.8	2.3	1.2

Chi-squared (X^{2}) test was applied. There was no significant difference between MI from smokers and non-smokers.

Table 3 Sister Chromatid Exchange (SCE) frequency was determined in 34 smokers

Individual	*Sister chromatid exchange*	
	Smoker	*Non-smoker*
Mean	2.98	2.87
SE	0.20	0.11

Student's *t*-test was applied. There was no significant difference between SCE from smokers and non-smokers.

Discussion

The genotoxicity of tobacco smoke was analyzed by measuring SCE frequencies. This variable was calculated for 44% of smokers tested ($n = 34$) and its value was not different from that of controls. We were not able to determine the genotoxic effect in the remaining 56% ($n = 43$) of the sample, because there were no second-division metaphases to allow SCE evaluation. Our finding for the smokers, where SCE could be determined, is in agreement with the reports of other authors, who did not find an elevated SCE frequency in smokers.[14–16,29,43] However, the majority of studies undertaken in the 1970's and 1980's reported a smoke-related increase in SCE frequency in smokers not exposed to other contaminants.[1,8–13,17,19,31,32,44–54]

There were significant correlations between age and number of years the subject had been smoking, as well as between RI and nicotine and cotinine levels and between CPK (M1, M2 and M3) and nicotine and cotinine levels. Similar results have been reported for other studies, in which these variables were mainly correlated with an increment in SCE frequency. More specifically, significant correlations were found between enhanced SCE frequency and number of cigarettes per day; enhanced SCE frequency and cigarette packets per year and between age and number of years the subject had been a smoker.[10,25,26,47,55] The results reported by Zhang *et al.*,[37] showing cell proliferation in human lung

cancer cells exposed to nicotine, are in agreement with the relationship between cotinine and RI found in the present study. It seems that the genotoxic and cytotoxic effects are more clearly found in those subjects that have been smokers throughout their lives.

The damage induced by exposure to tobacco smoke in smokers was evaluated by cytogenetic analysis by SCE, and by CPK and RI analysis. A cytostatic effect was shown by a delay in the cell cycle and a low-cell replication rate, with an average of 56.3 and 55.0 first division metaphases in moderate and heavy smokers respectively, and a decrease in RI. These results are in agreement with Barale *et al.*[26] who found a diminution in RI in heavy smokers (>20 cigarettes/day) and with the findings of Obe *et al.*[56] and Reidy *et al.*[12] However, the results of other studies are contradictory. For example, Husgafvel-Pursiainen *et al.*[11] found a decrease in M1 metaphase, an increase in M2 and M3, as well as in RI in active smokers ($n = 12$) when compared to non-smokers. Rupa *et al.*[57] reported a slight decrease in M1 and M2 metaphases in smokers ($n = 27$), as compared to non-smokers, but M3 metaphases were significantly increased in smokers. In both studies RI values indicated acceleration of the cell cycle and an increment in M1 in smokers. Both studies showed an increase in the cytostatic effect.

On the other hand, an increase in SCE induction and cell cycle delay and a diminution in mitotic index have been observed in workers that apply pesticide sprays who also smoke, compared with smokers that were not exposed to pesticides, indicating that smokers run a higher risk of genetic damage if also exposed to pesticides.[57] Similarly, other studies have reported significantly elevated SCE values among smokers exposed to occupational hazards compared similarly exposed non-smokers and/or to non-exposed smokers, for example smokers handling chemical solvents in various types of laboratories compared with non-smokers,[58] smokers working in contact with epoxy resins compared with non-smokers,[29] and smokers exposed to lead compared with non-exposed smokers. In the last group, metal-induced chromosomal damage results in a delay in blast transformation and consequently the majority of lymphocytes were still in first-division metaphase in cell cultures after 72 h.[59–62] An increase in SCE was also reported in smokers exposed to petroleum derivatives compared with non-exposed smokers.[63]

We found a positive correlation between high levels of nicotine in light and moderate smokers and CPK (delay in the cell cycle), as well as between moderate smokers and RI, which indicates that there is an acute effect caused by nicotine, considering its short half-life.[33] However, the positive correlation found between nicotine levels in moderate and intense smokers and the delay in CPK and the decrease in RI, suggests a chronic effect, as the half-life of this metabolite is seven times greater than nicotine.[33] This could be the result of the oxidative damage to DNA and/or of its low repair capacity, shown by the delay or decrease in cellular proliferation (CPK and RI) of normal cells[64,65] in which an induction of an apoptic process has also been observed. In contrast, cancerous and tumor cells show an increase in their cell proliferation,[37,64,66] in these types of cells a decrease in the apoptic process has also been observed.

Summarizing, the delay in CPK and the diminution in RI may be responses not only to oxidative damage, consistent in one and two-stranded DNA fragmentation, but also to nicotine exposure. This damage has also been observed in a rat lymphocytes study that was conducted on a group treated with nicotine using anti-oxidative compounds: a significant increase in the levels of lipid peroxidative index (TBARS and hydroperoxides (HP)) was reported, as well as an increase in the severity of the oxidative damage to DNA. This damage was evaluated by comet assay. These effects were significantly decreased in groups treated with ferulic acid and *N*-acetylcysteine.[36] This same effect was observed using ellagic acid in the same biological model when the DNA response was assessed by comet and micronuclei assays.[67]

Alternatively the CPK delay and RI decrease may be caused by a possible chronic effect in DNA damage and/or in the inhibition of the DNA repair process, which by means of an additive effect, increases cell proliferation due to oxidative damage caused by nicotine and cotinine as shown by the results Fracasso *et al.*[68] Their results indicated high basal DNA damage with clear significant correlations with urinary nicotine and cotinine, and the number of cigarettes/day. 'The DNA repair capacity had seriously decreased in non-smokers > smokers > ex-smokers, while the same damage was repaired in a short time in subjects that never smoked'.[68] Our results show a significant inverse relationship between CPK and RI values, with respect to nicotine and cotinine urinary levels, which suggests a possible inhibition in the repair process of DNA.

In conclusion, cytokinetic and cytostatic effects were mainly detected in heavy and moderate smokers. The cell cycle delay and RI decrease found in all 'healthy' smokers (not suffering from smoking-related illnesses) were similar to the results of other authors who studied cytokinetic and genotoxic changes in smokers.[29,60–62,69]

On the other hand the nicotine and cotinine exposure (causing oxidative damage to DNA) may have implications in the decrease in cell replication due to direct damage to DNA and/or a decrease in the DNA repair mechanisms. Alternatively, nicotine and cotinine may possibly induce apoptosis.

Additionally the correlation observed between cotinine levels and cytostatic effects may be a very important for triggering a carcinogenic or apoptosis mechanism in chronically exposed cells.

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