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GENOTOXICITY OF COMMERCIAL PETROL SAMPLES IN CULTURED HUMAN LYMPHOCYTES

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ABSTRACT

Different concentrations of two petrol commercial samples, leaded and unleaded, were evaluated for genotoxicity in human peripheral blood lymphocyte cultures. Sister-chromatid exchanges (SCE) and micronuclei (MN) were scored as genetic endpoints to measure the genotoxicity of the samples in cultures set up from three different healthy donors. The treatment of the cell culture was done employing different petrol concentrations (from 0.019 to 0.312 µl/ml). From our results we can conclude that both petrol samples induced a slight but concentration-dependent increase in the frequency of SCE, while no increase was detected in the MN frequency.

RESUMEN

Se evaluaron dos muestras de diferentes gasolinas comerciales, con y sin plomo, en cuanto a su genotoxicidad en cultivos de linfocitos humanos de sangre periférica. Para ello se realizaron ensayos de intercambios de cromátidas hermanas (ICH) y de micronúcleos (MN), a partir de la sangre de tres donantes sanos, efectuándose el tratamiento de los cultivos con diferentes concentraciones de las muestras de gasolina (de 0.019 a 0.312 µl/ml). De los resultados obtenidos se desprende que ambos tipos de gasolina indujeron un incremento moderado, pero dependiente de la concentración, en la frecuencia de ICH, pero no así en la de MN.

INTRODUCTION

Petrol can be considered as one of the most common substances used by citizens of industrialized countries. It is refined from petroleum and marketed to millions of consumers whose mobile life style depends on it (MacGregor 1993). In addition, petrol can be used as a fuel, diluent, finishing agent and industrial solvent (Sittig 1984).

Automotive petrol is a complex mixture of relatively volatile hydrocarbons, with or without additives, obtained by blending appropriate refinery products. Different commercial petrol mixtures may have different compositions, depending on its origin and blending procedure. Petrols include leaded and unleaded grades, both of which are manufactured from blends of straight-run, cracked, reformed and other naphta streams. A typical composition of unleaded petrol is qualitatively similar to premium leaded grade but without lead antiknock additives (Hoffman 1982).

The intensive and extensive use of petrol today guarantees that a large proportion of human population is exposed to its effects at a high level (petrochemical and petrol attendance workers) or at low level (general population). Petrol acute toxicity studies indicate that it can be considered only moderately to mildly toxic (Reese and Kimbrough 1993); although epidemiological studies have demonstrated an elevated risk of leukemia and other blood diseases among workers exposed to petrol (Brandt *et al.* 1978, Flemming 1990, Infante *et al.* 1990). In addition, carcinogenicity studies in mice and rats seem to reveal an increase in the tumor frequency in treated animals (MacFarland *et al.* 1984). Until now it remains unclear which components of petrol are of most concern from the genotoxic point of view, since currently formulated petrol contains a myriad of compounds; nevertheless benzene has been considered by many authors as the most logical genotoxic indicator (Infante 1993), although other components of petrol such as diepoxybutane (Kelsey *et al.* 1995), 1,2-dibromoethane (Wormhoudt *et al.* 1996) and methyl tertiary butyl ether (Hutcheon *et al.* 1996) should also be considered.

Taking into account human population concerns about the genotoxic effects that petrol exposure may provoke, we report the effects that leaded and unleaded petrol commercial samples have on human peripheral blood lymphocytes genetic material by using the sister-chromatid exchanges (SCE) and micronuclei (MN) *in vitro* assays.

MATERIALS AND METHODS

Chemicals

Two commercial samples of petrol, leaded and unleaded, were obtained from two different pumps in Barcelona selected at random. Mitomycin C (MMC, CAS No 50-07-7), used as positive control, was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Petrol was dissolved in methanol (CAS No 67-56-1) and 0.05 ml of this solution was added to the culture media 24 hours after the initiation of the cultures, to obtain five different concentrations of petrol into the media, ranging from 0.019 to 0.312 µl/ml, which indicate the proportion of petrol in relation to the final volume of culture medium.

Lymphocyte cultures

Lymphocyte cultures were set up by adding 0.5 ml of heparinized whole blood to 4.5 ml of chromosome medium (RPMI 1640, Gibco) supplemented with 16% heat-inactivated fetal calf serum (Gibco), antibiotics (penicillin and streptomycin) and glutamine. Lymphocytes were stimulated by 4% phytohemagglutinin (Gibco). For each genetic endpoint, blood from different healthy non-smoking donors was used.

For sister-chromatid exchanges (SCE) demonstration, the cultures were incubated at 37°C for 72h and 5-bromodeoxyuridine (BrdU) at 15 µg/ml was added 24h after the initiation of cultures. The test compounds were added together with the BrdU. All cultures were maintained in total darkness to minimize photolysis of BrdU.

For the micronucleus (MN) study, the cultures were incubated at 37°C for 72h and, at 44h after their beginning, cytochalasin-B (Cyt-B) at a final concentration of 6 µg/ml was added to arrest cytokinesis. This concentration of Cyt-B is the highest concentration normally used and was selected because it gives a higher percentage of binucleated cells and a lower baseline MN frequency (Surrallés *et al.* 1992). The chemicals to test were added 24h after phytohemaglutinin stimulation.

In both assays, the treatment continued up to harvest and, for SCE, at 2 h prior to harvesting, 0.6 µg/ml of colcemid (Gibco) was added to arrest the cells at metaphase. None of the treatments produced significant pH changes in the culture medium.

The cells were collected by centrifugation and, for SCE demonstration, resuspended in a pre-warmed hypotonic solution (KCl 0.075 M) for 20 min and fixed in acetic acid : methanol (1:3, v/v). For MN, the cells were washed once in RPMI 1640 medium and then, a mild hypotonic treatment (2-3 min in KCl 0.075 M at room temperature) was carried out. Next, the cells were centrifuged and a methanol/acetic acid (5:1) solution was gently added. This fixation step was repeated twice and the resulting cells were resuspended in a small volume of fixative solution (Surrallés *et al.* 1992). Air-dried preparations were made and the slides were stained with 10% Giemsa in phosphate buffer for 20min (MN), or with fluorescence plus Giemsa (SCE) (Perry and Wolff 1974).

SCE and MN analysis

A total of 50 well spread second division metaphases in the SCE assay, and 1000 binucleated cells with well preserved cytoplasm in the MN assay were examined, when it was possible, for each experimental concentration and donor on coded slides.

In the SCE study, 100 metaphases per donor were also scored to determine the proportion of cells that underwent one, two and three or more divisions. The proliferative rate index (PRI) was calculated according to the formula PRI=(MI+2MII+3MIII)/N, where MI, MII and MIII indicate the numbers of metaphases in first, second and third or subsequent divisions and N the total number of metaphases scored (Lamberti *et al.* 1983). In the MN study, a minimum of 500 lymphocytes were scored to evaluate the percentage of cells with 1, 2, 3, 4 or more than 4 nuclei. A cytokinesis block proliferation index (CBPI) was calculated according to Surrallés *et al.* (1995) as follows: CBPI = [MI+2MII+3(MIII+MIV)]/N, where MI-MIV represent the number of cells with one to four nuclei, respectively, and N is the total number of cells scored.

For the statistical evaluation we used the *t*-test for SCE, the chi-square test for PRI and CBPI, the Fisher exact probability test for BNMN (binucleated cells with MN), and the Kastenbaum and Bowman test (1970) for MN.

RESULTS AND DISCUSSION

The ability of two commercial petrol samples to induce SCE has been investigated in blood cultures from three young healthy donors and the results are indicated in **tables I-III**. These data indicate that both petrol samples, leaded and unleaded, have a moderate genotoxic potential, inducing a slight but concentration-dependent increase in the frequency of SCE in the lymphocytes of three donors. However it should be recalled that, whatever the statistical approach used for the interpretation of SCE data, it is argued that a twofold increase in SCE over that of the concurrent control should be required for considering a positive result as an indication of clear genotoxic effects (Perry *et al.* 1984). On the other hand, a significant reduction in the PRI value was found, at least at the higher concentration tested (0.312 µl/ml).

MN induction has been studied in whole-blood cultures from two donors and the results obtained are summarized in **tables IV-V**. These results indicate a lack of response in both samples, the total number of MN and the number of binucleated

TABLE I. INDUCTION OF SCE AND CELL CYCLE DELAY BY SAMPLES OF COMMERCIAL PETROL IN CULTURED HUMAN LYMPHOCYTES

Donor	Conc.	Cells scored	SCE/cell ± SE	M1	M2	M3	PRI
A	**Control**	50	9.20 ± 0.65	43	47	10	1.67
	Methanol (10µl/ml)	50	9.38 ± 0.52	34	56	10	1.76
	MMC (0.2µM)	50	31.40 ± 1.07***	42	53	5	1.63
	Leaded petrol (µl/ml)						
	0.019	50	8.50 ± 0.44	38	53	9	1.71
	0.039	50	9.04 ± 0.56	45	46	9	1.64
	0.078	50	10.62 ± 0.53*	41	48	11	1.70
	0.156	50	11.42 ± 0.65**	41	47	12	1.71
	0.312	50	14.12 ± 0.82***	42	57	1	1.59*
	Unleaded petrol (µl/ml)						
	0.019	50	9.68 ± 0.51	24	56	20	1.96
	0.039	50	7.78 ± 0.50	26	54	20	1.94
	0.078	50	10.64 ± 0.54*	42	51	7	1.65
	0.156	50	12.18 ± 0.64***	35	49	16	1.81
	0.312	40	13.88 ± 0.64***	54	39	7	1.53*

Conc., concentration; SE, standard error; PRI, proliferative rate index

*P < 0.05; **P < 0.01; ***P < 0.001 (*t*-test for SCE; chi-square test for PRI). The significance is determined with respect to the control with methanol

cells with MN (BNMN), since the two slightly positive results obtained in cultures from donor B can be considered as marginal. In addition, the CBPI decreased with increasing concentrations of petrol, reflecting its cytotoxic effects.

Considering that the SCE assay is a well known test for detecting primary DNA damage and that the MN test appears to be a pertinent tool to demonstrate the induction of clastogenic and/or aneugenic effects (Ramalho *et al.* 1988, Eastmond and Tucker 1989), our experimental data allowed the detection of a wide spectrum of genetic damage induced by petrol in human lymphocytes.

Until now only a few studies have been conducted to evalute the genetic and related effects of petrol. From the available results it appears that commercial petrol is able to exert mutagenic activity in *Drosophila* by inducing somatic mutation in the UZ system after larval feeding in culture medium

TABLE II. INDUCTION OF SCE AND CELL CYCLE DELAY BY SAMPLES OF COMMERCIAL PETROL IN CULTURED HUMAN LYMPHOCYTES

Donor	Conc.	Cells scored	SCE/cell ± SE	M1	M2	M3	PRI
B	**Control**	50	8.66 ± 0.53	16	49	35	2.19
	Methanol (10µl/ml)	50	10.44 ± 0.49	28	3	36	2.08
	MMC (0.2µM)	37	26.81 ± 1.22***	31	32	37	2.06
	Leaded petrol (µl/ml)						
	0.019	50	9.10 ± 0.51	25	54	21	1.96
	0.039	50	11.28 ± 0.60	29	36	35	2.06
	0.078	50	10.44 ± 0.61	38	30	22	1.94
	0.156	50	13.30 ±0.70***	43	30	27	1.84
	0.312	50	-	48	11	0	1.19***
	Unleaded petrol (µl/ml)						
	0.019	50	11.62 ± 0.63	19	49	32	2.13
	0.039	50	10.24 ± 0.52	20	47	33	2.13
	0.078	50	10.68 ± 0.57	25	47	28	2.03
	0.156	50	10.84 ± 0.65	26	44	30	2.04
	0.312	48	12.73 ± 0.74**	50	47	3	1.53***

Conc., concentration; SE, standard error; PRI, proliferative rate index

*P < 0.05; **P < 0.01; ***P < 0.001 (t-test for SCE; chi-square test for PRI). The significance is determined with respect to the control with methanol

TABLE III. INDUCTION OF SCE AND CELL CYCLE DELAY BY SAMPLES OF COMERCIAL PETROL IN CULTURED HUMAN LYMPHOCYTES

Donor	Conc.	Cells scored	SCE/cell ± SE	M1	M2	M3	PRI
C	**Control**	50	7.24 ± 0.51	10	27	63	2.53
	Methanol (10µl/ml)	50	8.82 ± 0.47	17	35	48	2.31
	MMC (0.2µM)	50	32.96 ± 0.97***	45	49	6	1.61***
	Leaded petrol (µl/ml)						
	0.019	70	10.33 ± 0.53*	26	55	19	1.93***
	0.039	50	11.44 ± 0.66**	27	64	9	1.82***
	0.078	50	11.28 ± 0.68**	29	51	20	1.91***
	0.156	50	11.94 ± 0.60***	32	57	11	1.79***
	0.312	-	-	-	-	-	-
	Unleaded petrol (µl/ml)						
	0.019	29	9.45 ± 0.66	40	52	8	1.68***
	0.039	50	12.02 ± 0.61***	59	36	5	1.46***
	0.078	50	11.50 ± 0.53***	40	53	7	1.67***
	0.156	60	12.80 ± 0.71***	42	49	9	1.67***
	0.312	39	12.74 ± 0.98***	59	40	1	1.42***

Conc., concentration; SE, standard error; PRI, proliferative rate index

*P < 0.05; **P < 0.01; ***P < 0.001 (*t*-test for SCE; chi-square test for PRI). The significance is determined with respect to the control with methanol

containing leaded petrol (Nylander *et al.* 1978). In addition, unleaded petrol induced mutations in mouse lymphoma L51784Y $TK^{+/-}$ cells as it has been reported in an abstract by Farrow *et al.* (1983) and in the paper by Dooley *et al.* (1988), in which positive results were obtained after metabolic activation.

On the other hand, unleaded petrol failed to induce revertants in the Ames test performed with and without metabolic activation (Conaway *et al.* 1983) and it was also negative in the induction of gene mutation, at the TK6 locus, and SCE in human lymphoblast cultures, with and without

TABLE IV. INDUCTION OF MICRONUCLEI AND CYTOTOXICITY BY 48-H TREATMENT WITH COMMERCIAL PETROL IN HUMAN WHOLE-BLOOD LYMPHOCYTE CULTURES

Donor	Treatment	Distribution of BN cells according to No. MN					MN	BNMN	Distribution of cells according to No. nuclei				%BN	CBPI
		0	1	2	3	>3			1	2	3	4		
A	**Control**	996	4	0	0	0	4	4	89	362	19	30	72.4	1.92
	Methanol (10µl/ml)	989	11	0	0	0	11	11	108	345	11	36	69.0	1.88
	MMC (0.4 µM)	914	83	3	0	0	89***	86***	148	327	8	17	65.4	1.75***
	Leaded petrol (µl/ml)													
	0.019	988	11	1	0	0	13	12	122	349	10	19	69.8	1.81
	0.039	993	7	0	0	0	7	7	144	331	4	21	66.2	1.76
	0.078	987	12	0	0	1	16	13	144	341	6	9	68.2	1.74***
	0.156	991	7	1	1	0	12	9	185	314	0	1	62.8	1.63***
	0.312	T	-	-	-	-	-	-	-	-	-	-	-	-
	Unleaded petrol (µl/ml)													
	0.019	993	4	2	0	1	12	7	119	341	14	26	68.2	1.84
	0.039	992	8	0	0	0	8	8	121	335	6	38	67.0	1.85
	0.078	993	7	0	0	0	7	7	140	337	8	15	67.4	1.77***
	0.156	990	8	1	1	0	13	10	218	278	3	1	55.6	1.57***
	0.312	T	-	-	-	-	-	-	-	-	-	-	-	-

T, 100% toxicity; MN, total MN; BNMN, binucleated cells with MN; %BN, percentage of BN cells; CBPI, cytokinesis blocked proliferation index. Probabilities: *, **, *** = P < 0.05, 0.01, 0.001 respectively (Fisher's exact test and Kastenbaum and Bowman test for BNMN and MN, respectively; chi-square test for CBPI). The significance is determined with respect to the control with methanol

TABLE V. INDUCTION OF MICRONUCLEI AND CYTOTOXICITY BY 48-H TREATMENT WITH COMMERCIAL PETROL IN HUMAN WHOLE-BLOOD LYMPHOCYTE CULTURES

Donor	Treatment	Distribution of BN cells according to No. MN					MN	BNMN	Distribution of cells according to No. nuclei				%BN	CBPI
		0	1	2	3	>3			1	2	3	4		
B	**Control**	986	12	2	0	0	16	14	63	360	34	43	72.0	2.03
	Methanol (10µl/ml)	989	10	10	0	0	12	11	109	345	26	20	69.0	1.87
	MMC (0.4 µM)	935	63	1	1	0	68***	65***	228	244	15	13	48.8	1.60***
	Leaded petrol (µl/ml)													
	0.039	706	16	2	0	1	25**	19*	299	164	18	19	32.9	1.48***
	0.039	990	10	0	0	0	10	10	79	380	19	22	76.0	1.92*
	0.078	991	9	0	0	0	9	9	97	373	13	17	74.6	1.87
	0.156	992	7	1	0	0	9	8	138	349	6	7	69.8	1.75***
	0.312	T	-	-	-	-	-	-	-	-	-	-	-	-
	Unleaded petrol (µl/ml)													
	0.019	984	15	1	0	0	17	16	321	163	10	6	32.6	1.39***
	0.039	986	14	0	0	0	14	14	218	253	17	12	50.6	1.62***
	0.078	986	13	1	0	0	15	14	179	287	19	15	57.4	1.71***
	0.156	987	13	0	0	0	13	13	132	355	6	7	71.0	1.76***
	0.312	733	16	2	0	0	20*	18*	369	127	3	12	25.4	1.27***

T, 100% toxicity; MN, total MN; BNMN, binucleated cells with MN; %BN, percentage of BN cells; CBPI, cytokinesis blocked proliferation index. Probabilities: *, **, *** = $P < 0.05, 0.01, 0.001$ respectively (Fisher's exact test and Kastenbaum and Bowman test for BNMN and MN, respectively; chi-square test for CBPI). The significance is determined with respect to the control with methanol

metabolic activation (Richardson *et al.* 1986). Moreover, in rat bone marrow following *in vivo* exposure, unleaded petrol failed to produce chromosomal aberrations (Conaway *et al.* 1983).

In studies carried out to check the ability of petrol to induce UDS in rat hepatocytes, a weak activity was observed in hepatocytes isolated from male and female mice treated with unleaded petrol, and a positive induction was also found after *in vitro* treatment (Loury *et al.* 1986).

From our positive findings in the SCE assay it appears that there is at least one genotoxic compound in the commercial petrol samples analyzed, which can be detected in human lymphocytes treated *in vitro*. Commercial petrol contains different hydrocarbons as well as several aromatic compounds, haloalkanes and lead, and several authors have indicated that some of them are genotoxic, i.e., 1,2-dibromoethane (Vogel and Chandler 1974), 1,2-dichloroethane (McCann *et al.* 1975), 2,2,4-trimethylpentane (Richardson *et al.* 1986) and benzene (Infante 1993); nevertheless, our interest is not in identifying the component(s) responsible for the petrol genotoxicity. It is known that the extraction procedures may either produce or destroy mutagenic species (Epler 1980) and the eventual interactions, antagonistic or synergistic, taking place between the different components of petrol can be missed.

The fact that our positive results have been obtained without the use of an external metabolic fraction seems to indicate that benzene is not the main genotoxic agent, since this compound requires metabolic activation to be genotoxic (Dean 1985), hydroquinone and catechol being the metabolites that are effective in inducing genetic damage in human lymphocytes (Robertson *et al.* 1991). Nevertheless, it must be recalled that human lymphocytes possess a certain metabolic capacity (Ikeuchi and Sasaki 1981).

On the other hand, long treatments as for instance 48 hours may allow the evaporation of the most volatile components of petrol and, consequently, the less volatile components could be the ones responsible for the detected genotoxicity. Furthermore, the fact that genotoxicity was detected both in leaded and unleaded samples seems to indicate that the addition of lead is not the cause of the eventual genotoxic effects of petrol.

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