Biomarkers of genotoxicity measured in human lymphocytes exposed to benzo[a]pyrene: Aneugenic effect, and involvement multiple primary DNA lesions

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Abstract

Benzo[a]pyrene (B[a]P) is a polycyclic aromatic hydrocarbon classified as carcinogenic to human. Its metabolic activation leads to production of metabolites forming adducts with DNA, being at origin of B[a]P-induced DNA damages, mutagenesis and carcinogenesis. Human blood lymphocytes cultures established from 25 subjects aged 20 to 30 years old, living in Montréal (Québec, Canada) were exposed to B[a]P (0.4, 4, 20 and 40 µM) for 24 h, then washed and cultivated without B[a]P for an additional 24 h. B[a]P-DNA adducts, DNA single-strand breaks (SSBs), sister chromatid exchanges (SCEs), chromosomal aberrations (CAs) and micronuclei (MN) were analysed. Fluorescent in situ hybridization (FISH) analysis of MN using a pancentromeric probe was also done to assess MN content. Significantly increased formation of B[a]P-DNA adducts, CAs, MN and SCEs were observed starting at [B[a]P]=0.4 µM (p<0.01), with a significant decrease of B[a]P-DNA adducts and MN after exposure to 20 µM B[a]P. For DNA SSBs, no significant increase was observed in all conditions. Besides, FISH analysis showed that B[a]P-induced MN mostly contain centromeres (77.1% vs 68.5% for the control, p<0.01), and specifically three or more centromeres (p<0.01). This study suggests an aneugenic effect of B[a]P in human lymphocytes. Finally, statistical analysis by multiple linear regression showed that two variables (B[a]P exposure level and B[a]P-DNA adducts) significantly explained 53% of observed variability in SCE test, while the percentage of cell presenting a high frequency of SCE (% HFC) was the only variable significantly explaining the observed variability in CA and MN test (34% and 12%, respectively). These observations indicate that DNA breaks, in addition to B[a]P-DNA adducts, contributed to SCEs formation.

Introduction

Benzo[a]pyrene (B[a]P) is a polycyclic aromatic hydrocarbon (PAH) produced during the incomplete combustion of organic matter and is ubiquitously present in our environment. PAHs are common contaminants of ambient air through cigarette smoke and air pollution, as well as a food contaminant. Occupational exposure to PAHs occurs in coal gasification and aluminium production industries, as well as during use of coal tar, asphalt and mixtures of coal-tar pitch [1]. Currently, 15 PAHs are classified as carcinogenic, probably or possibly carcinogenic to human by the International Agency for Research on Cancer (groups 1, 2A and 2B) [1], and B[a]P is a prototypical member of this class of carcinogenic PAHs.

Bioactivation of B[a]P leads to production of r7,18-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydronbenzo[a]pyrene (BPDE), which is considered as the ultimate carcinogenic species. BPDE can bind to DNA, forming adducts that will be removed mainly by nucleotide excision repair (NER) [2]. The major stable adduct of B[a]P is the BPDE adduct (r7,18,9-trihydroxy-c-10-(N2-deoxyguanosyl)-7,8,9,10-tetrahydronbenzo[a]pyrene). In addition, B[a]P quinone metabolites can form stable and depurinating adducts with DNA. It can be re-oxidized, thus generating reactive oxygen species (ROS), which will oxidize DNA bases generating 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-OH-dG) [3]. These adducts are at the origin of B[a]P-induced DNA damage, mutagenesis and carcinogenesis [4,5], and their levels are increased in occupationally exposed individuals presenting raised levels of cytoxic biomarkers such as sister chromatid exchanges (SCEs), chromosome aberrations (CAs) and micronuclei (MN) [6-10]. As for DNA damage measured with the Comet assay, some studies report a significant increase in PAH exposed workers [11,12], while other studies did not report such an increase [13,14]. In order to...
evaluate DNA damage, biological monitoring is commonly performed on human lymphocytes, used as surrogate tissue, as they are considered to reflect damage present in target tissues [15].

In vitro exposure of human lymphocytes to B[a]P permitted the characterization of B[a]P-DNA adducts by 3P-postlabeling [16,17], however failed in measuring DNA single-strand breaks with the Comet assay, following a short exposure period to B[a]P (1 or 4 h; 0.5–10 µM B[a]P) [18,19]. As for early biomarkers of genotoxicity, a significant production of SCEs was observed in lymphocytes treated with up to 250 µM B[a]P for a 48h exposure period [20-22]. Also, a significant production of MN was found in lymphocytes treated with up to 15 µM B[a]P for long exposure periods (72 and 64 h) [23,24], while for CAs significant increases were observed in lymphocytes treated with B[a]P for shorter periods (24 or 48 h) [20,25]. Evidences of B[a]P metabolism in human lymphocytes first came from quantification of BPDE and quinones [16], then from gene expression analyses showing presence of proteins involved in phase I and phase II metabolism of B[a]P (CYP1A1 and GSTP1), and in its induction (AhR, ARNT) [26]. Together with the fact that CYP1A1 enzyme is induced in benzoanthracene treated lymphocytes [27], these studies suggest that human lymphocyte culture is a metabolically competent cell system and, therefore, is a good model for in vitro studies of B[a]P-induced genotoxicity.

MN and CA tests can be used to identify the properties a particular compound. MN originate mainly from chromosome fragments or whole chromosome lagging at anaphase during cell division reflecting, respectively, clastogenic and aneugenic events [28]. As for CAs, chromatid- and chromosome-type aberrations reflect clastogenic events [29]. Following evaluation of the MN test by the GUM (a German speaking section of the European Environmental Mutagen Society) and the ECVAM (European Centre for the Validation of Alternative Methods) [30,31], B[a]P has been classified as a clastogen. However, reported MN studies on PAH or B[a]P metabolism is a metabolically competent cell system and, therefore is a good model for in vitro studies of B[a]P-induced genotoxicity.

In this study, we evaluated the genotoxicity of B[a]P on human lymphocytes exposed to low B[a]P concentrations (0.4–40 µM) with a panel of selected biomarkers. Our goals were to evaluate the effect of B[a]P exposure on the levels of B[a]P-DNA adducts, DNA single-strand breaks (DNA SSBs) and cytogenetic biomarkers, and to confirm the clastogenic effect of B[a]P on human lymphocytes. We measured B[a]P-DNA adducts and DNA single strand breaks (SSBs) as primary DNA lesions. Cytogenetic effects of B[a]P were evaluated with SCE test, CA test, and MN assay, while MN content was assessed with a FISH-coupled MN assay. Finally, we studied correlations between the biomarkers, and examined sex differences, as they are only rarely assessed in occupational and environmental studies. To our knowledge, very few reports have assessed such a large number of early biomarkers following B[a]P exposure of human lymphocytes.

Materials and methods

Subjects

Twenty five subjects (13 women and 12 men), aged 20 to 30 years old (mean: 26.6 years), living in Montréal (Québec, Canada) were carefully selected to ensure minimal exposure to PAHs and other known genotoxic agents. PAH intake through the diet was not documented and blood collection of all subjects was performed during an entire year. All subjects were healthy non-smoking students at
well, as there was a limited amount of cells available for culture. Resulting in each sample DNA being measured in one experimental and per subject was prepared for B[a]P-DNA adducts measurements, resulting in each sample DNA being measured in one experimental well, as there was a limited amount of cells available for culture.

DNA SSB measurements with the Electron Microscopy In Situ End-labeling (EM-ISEL) assay

Colcemid (0.1 mg/ml – Invitrogen Canada Inc.) was added to cultures 2 h prior harvesting cells in metaphase. Cells were centrifuged, resuspended in 0.075 M KCl, incubated for 8 minutes at 37°C, centrifuged and fixed three times in Carnoy I. Finally, metaphase cells were spread on ice-cold precleared microscope slides and air-dried overnight at room temperature. DNA SSBs were detected using the EM-ISEL assay, as described in Depault et al. [37]. One culture per treatment and per subject, was analysed with EM-ISEL assay, due to limitation in blood volume. Twenty nuclei were observed and photographed with a Philips EM208 transmission microscope (Philips Electron Optics, Eindhoven, The Netherlands) at 36000x of magnification. Labeling density was evaluated by counting the number of immunogold particles (IGPs) per µm² of chromatin using the public domain software Scion Image for Windows, version 4.0.3.2 (Scion Corporation, Frederick, MD, USA).

Sister chromatid exchanges (SCE) test

BrdU (10µM – Sigma-Alrich Canada Ltd) was added to cultures for the last 24 h of culture. Colcemid (0.1 mg/ml – Invitrogen Canada Inc.) was added to cultures 2 h prior harvesting cells in metaphase. Cells were centrifuged, resuspended in 0.075 M KCl, incubated for 8 minutes at 37°C, centrifuged and fixed three times in Carnoy I. Finally, metaphases were spread on ice-cold precleared microscope slides. Fluorescence-Photolysis-Giemsia (FPG) technique was then performed to visualize sister chromatids and SCEs [38]. Two cultures per treatment and per subject were analyzed. For each culture, 25 M2 metaphases were analyzed at 1000x of magnification on a Leica Aristoplan microscope (Leica Microsystems Canada Inc.), for SCE number. Selected metaphases contained 46 chromosomes, in accordance with OECD guideline 479 [39]. All microscopic analyses were done by a single observer. High frequency SCE cells (HFC) were evaluated following a modification of the method described by Kosmider et al. [40]. The number of SCE corresponding to the 95th percentile of SCE distribution ([B[a]P]=0 µM) was determined for each subject. The percentage of cells having more SCEs than the 95th percentile was calculated for all subjects and all conditions.

Micronuclei (MN) assay

Cytokinesis-block MN assay was done following Fenech [28], with minor changes. 6 µM (final) cytochalasin-B was added to the cultures for the last 24 h of culture, before cell harvest. Cells were centrifuged, resuspended in 0.075 M KCl (Sigma-Aldrich Canada Ltd), incubated for 5 minutes at room temperature, centrifuged and fixed twice in Carnoy 1 (methanol: glacial acetic acid – 3:1 v/v, Fisher Scientific Company, Ottawa, ON, Canada). Finally, cells were spread on precleared microscope slides (Fisher Scientific Company) and Giemsa staining was realized with freshly filtered 2% Giemsa diluted in Gurr’s pH 6.8 buffer (both from Invitrogen Canada Inc.). Two cultures per treatment and per subject were analyzed. For each culture, 1000 binucleated (BN) cells with well preserved cytoplasm were analysed at 1000x magnification on a Leica Laborlux microscope (Leica Microsystems Canada Inc., Richmond Hill, ON, Canada). BN cells were selected and MN were analysed following the Fenech criteria [28]. Also, nuclear division index (NDI) was determined for all BaP conditions, by scoring the number of cells containing one (N1), two (N2), three (N3) or four (N4) nuclei on 1000 cells, as described in Fenech [28]. All slides were analysed by the same observer.

Chromosome Aberration (CA) test

For the CA test, colcemid (0.1 mg/ml – Invitrogen Canada Inc.) was added to cultures 2 h prior harvesting to arrest cells in metaphase. Cells were centrifuged, resuspended in 0.075 M KCl, incubated for 8 minutes at 37°C, and then fixed three times in Carnoy I. Finally, metaphases were spread on ice-cold precleared microscope slides, and Giemsa staining was realized with freshly filtered 3% Giemsa diluted in Gurr’s pH 6.8 buffer. Two cultures per treatment and per subject were analyzed. For each culture, 100 well spread metaphases were analysed at 1000x magnification on Leica Laborlux and Aristoplan microscopes (Leica Microsystems Canada Inc.). Chromosome number, gaps, breaks and complex aberrations were recorded. Cells with 46 ± 2 chromosomes were chosen for CA analysis, in accordance with OECD guideline 473 for the in vitro mammalian chromosome aberration test [41]. Cells with chromosome losses and gains were also recorded (43 ≤ chromosomes ≤ 49, triploid and tetraploid cells, as well as androduplications). Also, mitotic index (MI) was determined for all BaP conditions, by scoring the number of metaphases and nuclei on 1000 cells. All microscopic analyses were done by two observers, one duplicate culture per observer, for all subjects.

FISH-coupled MN assay

A pancentromeric probe (Cambio, Cambridge, UK) was used to assess the centromeric (Cen) content of MN for both the negative control and the [B[a]P]=20 µM conditions. For each condition, a slide for both conditions was stained with Giemsa; all MN present in BN cells were preselected, and their positions carefully recorded on a Leica Aristoplan dual photonic/fluorescence microscope (Leica Microsystems Canada Inc.) before hybridization. After destaining with Carnoy I, slides were treated with RNase A and pepsin (both from Sigma-Aldrich Canada Inc.) to remove endogenous RNA and cytoplasmic proteins. This step ensured an optimal penetration of the probe during hybridization, and thus an optimal visualization of Cen signals in MN. Hybridization was performed following manufacturer’s instructions and slides were counterstained with DAPI (Roche Diagnostics, Laval, QC, Canada) before microscopic analysis. Positions of preselected MN were retrieved and number of Cen signals were recorded, for both the negative control and the [B[a]P]=20 µM condition. Only MN where the FISH signals were clearly visible and homogeneously dispersed within the BN cell wereanalysed.

Statistical analysis

For DNA SSBs (n=20 per subject and treatment) and SCEs (n=50 per subject and treatment), raw data were analysed on an individual level by one-way ANOVA followed by multiple comparison post-hoc test (Bonferroni for equal variances or Tamhane’s T2 for unequal
variances). To perform statistical analysis at the group level, DNA SSB data were expressed as means normalized to the negative control, while SCE data were expressed as mean SCEs per cell, and as percentage HFC. For MN, tested indicators were: frequency of MN in BN cells, and number of MN per micronucleated BN cells (MN per MNBN). For CAs, tested indicators were: frequency of cells presenting one or more CA (CA cells), frequency of CA and percentage of cells with chromosome losses and gains. Dose-response relationships of B[a]P-DNA adducts and DNA SSBs were analysed with the non-parametric Wilcoxon signed-Rank test. For CA, MN, SCEs and % HFC, repeated measures ANOVA were performed. Inclusion or exclusion of gaps during statistical analysis did not change the results.

For analysis of MN content by FISH, MN were classified for the two conditions (negative control and [B[a]P]=20 µM) in the following categories, as suggested by Iarmarcovai et al. [33]. First, MN containing no Cen signal (C-MN), and containing 1 or more Cen signals (C+ MN) were enumerated. Second, C+ MN were divided in two new categories according to the number of Cen signals present: C1-2+ MN (1-2 Cen signals) and C+ MN (3 or more Cen signals). The two-proportion z-test for independent samples was used to test if proportion of C+ MN, a human lymphocytes with increasing B[a]P exposure. No significant decreases were observed following B[a]P and did not compromise results of our genotoxicity testing. This B[a]P-DNA adduct level, as adducts were non detected were assigned a value corresponding to half B[a]P-DNA adduct measurements by CIA.

To study the relationships existing between the different biomarkers, Pearson’s correlation, as well as partial correlation (adjusted on B[a]P exposure and sex), were performed. Also, multiple linear regression, testing for collinearity, was conducted to explain the variability observed in our biomarkers. Samples in which B[a]P-DNA adducts were non detected were assigned a value corresponding to half the limit of detection (LOD/2). Statistic analyses were done using SPSS 17.0 software for Windows (SPSS inc. Chicago, IL, USA), and p-values less than 0.05 were considered statistically significant.

**Results**

Preliminary experiments were done to determine lymphocyte viability (using Trypan blue exclusion assay) following 24 h exposure to 0–0.4–4–20 and 40 µM B[a]P. These experiments, performed on 4 subjects (2 females and 2 males) showed that more than 80% cells were viable after B[a]P exposure (data not shown). Also, analysis of NDI showed a significant decrease when cells were exposed to 40 µM B[a]P, compared to negative control (Table 1), suggesting the presence of a cytostatic effect. However, with an observed NDI of 1.60 ± 0.05 at B[a]P=40 µM, it is well above the lower threshold value of 1.3 cited by Fenech [28], indicating that viability of exposed cells was adequate, and did not compromise results of our genotoxicity testing. This was further confirmed by analysis of the mitotic index (Table 1), as no significant decreases were observed following B[a]P exposure. Together, these results reveal a trend towards a reduction in viability of human lymphocytes with increasing B[a]P exposure.

**B[a]P-DNA adduct**

Individual results for B[a]P-DNA adduct measurements by CIA are presented in Table 5, while group mean values are in Table 2. Interindividual variation was present when measuring the spontaneous ([B[a]P]=0 µM) B[a]P-DNA adduct level, as adducts were non detected in 76% of the unexposed samples (Table 5). Comparison at group level showed a significant increase in B[a]P-DNA adducts following B[a]P exposure, starting at [B[a]P]=0.4 µM (p<0.001 for the group –Table 2). Maximal B[a]P-DNA adduct level was observed at [B[a]P]=4 µM, followed by a significant decrease at [B[a]P]=20 µM, for both group (p<0.05), and women (p<0.01). Direct comparison of women and men results in all B[a]P-tested conditions showed no significant differences, despite the 1.6 fold difference at B[a]P=20µM condition (Table 2).

**DNA single-strand breaks (DNA SSBs)**

Statistical analysis at an individual level by one-way ANOVA showed significantly increased IGPs for 4/10 subjects at [B[a]P]=0.4 µM, when compared to negative control (p<0.001 – Table 6). At [B[a]P]=4 µM, differences between individuals were especially present, as 5/10 subjects presented a significant increase in all B[a]P conditions tested. Direct comparison of women and men results in all B[a]P-tested conditions showed no significant differences.

**Sister chromatid exchanges (SCEs)**

Group analysis shows that significant increases in mean SCEs per cell and % HFC were observed following B[a]P exposure, starting at [B[a]P]=0.4 µM (p<0.05–Table 2). These increases were linear: individual mean SCEs per cell (data not shown) and individual % HFC (Table 7) presented a very good correlation with B[a]P exposure (Pearson’s r=0.64 and r=0.66; p=1.5 x 10⁻⁴ and p=2.9 x 10⁻⁴, respectively – Table 4). However, men and women behaved differently (see Table 2), as women showed a non-significant increase in mean SCEs per cell at [B[a]P]=0.4 µM, and a significant increase at [B[a]P]=4 µM (p<0.05), while men presented highly significant mean SCE increases at both concentrations (p<0.001). When analyzing % HFC, the same situation was observed (Table 2); men had significantly increased % HFC at [B[a]P]=0.4 and 4 µM (p<0.05 and p<0.001, respectively), while women presented significant % HFC increases starting at [B[a]P]=4 µM (p<0.05).

**Chromosome Aberration (CA) analysis**

B[a]P exposure caused mainly gaps and chromatid breaks, but
Table 2. Biomarkers of genotoxicity ([B(a)P]-DNA adducts, DNA SSBs, mean SCEs per cell, percentage of HFC, MN frequency, MN per MNBN, CA frequency, percentage of cells presenting chromosome gains (losses) measured in human lymphocytes drawn from healthy subjects, in vitro exposed to benzo[a]pyrene. Results expressed as means ± SEM.

| Biomarker | 0 | 0.4 | 4 | 20 | 40 |
|-----------|---|-----|---|----|----|
| [B(a)P]-DNA adducts (per 10^6 nucleotides) measured with the BPDE-DNA CIA assay | | | | | |
| Group (n=24) | 136 ± 54 | 6935 ± 891^†† | 14661 ± 1330^†† | 10760 ± 1951^†† | na |
| Women (n=15) | 115 ± 62 | 5792 ± 806^† | 13079 ± 1526^†† | 8324 ± 1596^† | na |
| Men (n=11) | 160 ± 95 | 8286 ± 1654^†† | 16383 ± 2185^†† | 13700 ± 3732^†† | na |

DNA SSBs (normalized mean) measured with the EM-ISEL assay:

| Group (n=10) | 1.00 | 1.37 ± 0.17 | 1.68 ± 0.34 | 1.28 ± 0.35 | 1.72 ± 0.19 |
| Women (n=5) | 1.00 | 1.43 ± 0.21 | 1.54 ± 0.61 | 1.29 ± 0.28 | 1.61 ± 0.45 |
| Men (n=5) | 1.00 | 1.32 ± 0.30 | 1.82 ± 0.37 | 1.26 ± 0.73 | 1.84 ± 0.01 |

Mean SCEs per cell determined during the SCE test

| Group (n=16) | 7.6 ± 0.12 | 11.1 ± 0.21^†† | 14.6 ± 0.22^†† | 16.2 ± 0.22^†† | na |
| Women (n=8) | 7.5 ± 0.15 | 10.7 ± 0.36 | 13.9 ± 0.31** | 16.0 ± 0.34^† | na |
| Men (n=8) | 7.8 ± 0.18 | 11.4 ± 0.22^†† | 15.3 ± 0.31 | 16.4 ± 0.30^†† | na |

HFC (%) determined during the SCE test

| Group (n=16) | 5.1 ± 1.75 | 25.9 ± 16.11^** | 57.8 ± 19.56^† | 66.9 ± 16.60^† | na |
| Women (n=8) | 5.8 ± 1.98 | 25.3 ± 18.39 | 56.6 ± 24.43^** | 68.6 ± 20.22^† | na |
| Men (n=8) | 4.5 ± 1.41 | 26.5 ± 14.73^** | 58.9 ± 14.83^†† | 65.4 ± 14.11^†† | na |

MN frequency (%) determined during the MN assay

| Group (n=20) | 9.9 ± 1.12 | 13.6 ± 1.56^** | 17.8 ± 1.80^†† | 20.9 ± 1.91^† | 17.9 ± 1.50^†† |
| Women (n=10) | 10.4 ± 1.8 | 12.8 ± 2.3^* | 18.8 ± 2.9^* | 21.1 ± 3.0^* | 18.7 ± 2.5^* |
| Men (n=10) | 9.4 ± 1.6 | 14.4 ± 2.0^** | 16.9 ± 2.2^†† | 18.9 ± 2.6^†† | 17.0 ± 1.6^†† |

MN per MNBN^‡ determined during the MN assay

| Group (n=20) | 1.061 ± 0.022 | 1.086 ± 0.020 | 1.135 ± 0.025^†† | 1.100 ± 0.022** | 1.083 ± 0.014 |
| Women (n=10) | 1.084 ± 0.040 | 1.076 ± 0.029 | 1.147 ± 0.033^§ | 1.086 ± 0.020 | 1.092 ± 0.021 |
| Men (n=10) | 1.037 ± 0.018 | 1.097 ± 0.029^§ | 1.124 ± 0.037** | 1.114 ± 0.040** | 1.074 ± 0.020 |

CA frequency (%) determined during the CA test

| Group (n=15) | 1.6 ± 0.3 | 3.6 ± 0.5^* | 4.4 ± 0.8^†† | 7.5 ± 1.0^* | 6.9 ± 1.6 |
| Women (n=8) | 2.1 ± 0.5 | 3.8 ± 0.5^* | 5.4 ± 1.4^* | 7.9 ± 1.6^* | 8.0 ± 3.5 |
| Men (n=7) | 1.1 ± 0.3 | 3.4 ± 0.8^* | 3.2 ± 0.8^* | 7.1 ± 1.4^* | 5.8 ± 0.8 |

CA cells (%) determined during the CA test

| Group (n=15) | 1.5 ± 0.2 | 3.3 ± 0.3^* | 3.6 ± 0.5^* | 6.6 ± 0.9^* | 5.8 ± 1.4^‡ |
| Women (n=8) | 1.8 ± 0.3 | 3.5 ± 0.3^* | 4.1 ± 0.7^* | 7.2 ± 1.5^* | 7.0 ± 3.0 |
| Men (n=7) | 1.1 ± 0.3 | 3.0 ± 0.5^* | 2.9 ± 0.7^* | 5.9 ± 1.1^** | 4.5 ± 0.5 |

Chromosome losses/gains (%) determined during the CA test

| Group (n=15) | 0.21 ± 0.06 | 0.57 ± 0.13** | 0.57 ± 0.09** | 0.87 ± 0.14^†† | 0.25 ± 0.02 |

na: not available - chromosome preparation not available
*non detected were assigned a value corresponding to half the limit of detection.
^ Mean number of MN per micronucleated cell (containing one or more MN)
†Percentage of cells presenting one or more CAs
‡Cells with 43≤ chromosomes ≥49
*p<0.1 **p<0.05 ††p<0.01 ‡‡p<0.001

chromosome breaks and complex aberrations were also recorded. Significant increases in CA cells and CA frequency (with and without gaps) were observed, starting at [B(a)P]=0.4 µM (group –p<0.01; Table 2). Maximal percentage of CA cells and CA frequency were observed at [B(a)P]=20 µM, followed by a slight decrease at [B(a)P]=40 µM. The CA cell decrease was moderate (non-significant) when compared to [B(a)P]=20 µM (p<0.01). We also observed a significant increase in cells presenting chromosome losses and gains, starting at [B(a)P]=0.4 µM (group: p<0.05 – Table 2). Direct comparison of women and men samples in all B(a)P-tested conditions (CA cells and CA frequency), showed no significant sex differences. On the other hand, dose-response relationship for CA frequency (Table 2) was different for the two sexes. Women behaved differently at [B(a)P]=0.4 µM, as they showed a moderate non-significant (p<0.01) increase in CA frequency, while men showed a significant increase (p<0.05).

Micronuclei (MN) analysis

An interindividual variation was present among the subjects with spontaneous ([B(a)P]=0 µM) MN frequencies per 1000 BN cells ranging from 3.5 to 21 for women, and from 1 to 17 for men (not shown), with a mean MN frequency of 9.9 ± 1.2 for the group (Table 2). While women had an increased spontaneous MN frequency when compared to men, it was non-significant. Following [B(a)P] exposure, significant increases in MN frequency was observed, starting at [B(a)P]=0.4 µM (p<0.01 – Table 2). Maximal MN frequency was observed at [B(a)P]=20 µM, followed by a decrease at [B(a)P]=40 µM. This decrease was significant only for men, when compared to [B(a)P]=20 µM (p<0.05), while the group showed only a moderate non-significant decrease (p<0.1). The number of MN per MNBN also increased following [B(a)P] exposure, being significant and maximal at [B(a)P]=4 µM (p<0.01 – Table 2).
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Table 3. FISH analysis of micronuclei (MN), using a pancentromeric probe, for [B[a]P]=0µM (negative control) and [B[a]P]=20µM (exposed condition).

| B[a]P (µM) | MN analyzed | C- MN (%) | C+ MN (%) | C1-2+ MN | C3++ MN (%) |
|------------|-------------|-----------|-----------|----------|-------------|
| Group      |             |           |           |          |             |
| 0          | 235         | 74 (31.5) | 161 (68.5)| 99 (71.7)| 80 (21.7)   |
| 20         | 477         | 109 (22.9)| 368 (77.1)| 77 (37.4)| 62 (62.6)   |
| Women      | 0           | 138       | 39 (28.3) | 99 (71.7)| 37 (37.4)   |
|            | 20          | 236       | 52 (22.0) | 184 (78.0)| 44 (23.9)   |
| Men        | 0           | 97        | 35 (36.1) | 62 (63.9)| 17 (27.4)   |
|            | 20          | 241       | 57 (23.7) | 184 (76.3)| 36 (19.6)   |

The number (and percentage) of C-, C+, C1-2+ and C3++ MN are presented for the group, as well as women and men separately. Percentages of C+ and C3++ MN present in the [B[a]P]= 20 µM condition were compared to the negative control condition using the two-proportion z-test.

'C': MN containing no centromere
'C+': MN containing one or more centromeres
'C1-2+': MN containing one or two centromeres
'C3++': MN containing 3 or more centromeres

*p-values are presented for negative control vs [B[a]P] exposure

Table 4. Pearson’s correlation coefficients and partial correlation coefficients (controlling for exposure and sex) existing between the biomarkers studied in human lymphocytes in vitro exposed to benzo[a]pyrene. Significant correlations are in bold.

| Biomarker | B[a]P exposure ¹ | Adducts (LOD/2)* | Mean SCEs per cell | Normalised SSBs | MN frequency | MN per MNBN | CA frequency |
|-----------|-----------------|------------------|-------------------|----------------|--------------|-------------|-------------|
|           | r=              | Adducts (LOD/2)* |                   |               |              |             |             |
|           | p=              |                   |                   |               |              |             |             |
|          | n=              |                   |                   |               |              |             |             |
| % HFC ²  | r=              | 0.664             | 2.9 x10⁻⁶         | 62            |              |             |             |
|           | p=              | 0.311             | 0.003             | 91            |              |             |             |
|          | n=              |                   |                   |               |              |             |             |
|          | r=              | 0.393             | 0.002             | 57            |              |             |             |
| Adducts ³ | p=              | 0.752             | 1.9 x10⁻⁶         | 61            |              |             |             |
|          | n=              | 0.022             | 0.012             | 26            |              |             |             |
|          |                  | 0.344             | 0.008             | 57            | 0.165        | 0.309       | 0.279       |
| Mean SCEs per cell ⁴ | p= | 0.308             | 0.007             | 34            | 0.281        | 0.160       | 0.235       |
|          | n=              | 0.188             | 0.079             | 26            | 0.214        | 0.170       | 0.345       |
| Normalised SSBs ⁵ | r= | 0.84              | 0.679             | 37            | 0.165        | 0.433       | 0.367       |
|          | p=              | 0.70              | 0.616             | 34            | 0.214        | 0.433       | 0.367       |
|          | n=              | 0.160             | 0.616             | 27            | 0.165        | 0.433       | 0.367       |
| MN frequency ⁶ | r= | 0.473             | 3.2 x10⁻⁹         | 87            | 0.099        | 0.160       | 0.180       |
|          | p=              | 0.074             | 0.604             | 51            | 0.180        | 0.307       | 0.351       |
| MN per MNBN ⁷ | r= | 0.351             | 0.011             | 51            | 0.351        | 0.011       | 0.351       |

slowly decreased at higher B[a]P concentrations, being significant for the group at [B[a]P]=40 µM, when compared to [B[a]P]=4 µM (p<0.05). Direct comparison of women and men samples in all B[a]P-tested conditions and for all MN assay indicators showed no significant differences. However, dose-response relationship for MN frequency was different for the two sexes. Women behaved differently at [B[a]P]=0.4 µM, as they showed a moderate non-significant increase (p<0.1 – Table 2) in MN frequency, while men showed a significant increase (p<0.05).

FISH-coupled MN analysis

Classification of FISH results in four categories (C-, C+, C1-2+ and C3++ MN) shows that B[a]P-induced MN were mostly C+ MN (p<0.01 – Table 3). Also, subdivision of C+ MN into two categories (C1-2+ and C3++ MN) revealed a proportional increase of MN containing three or more centromeric signals (C3++ MN), following B[a]P exposure (p<0.01). Sex analysis suggests that women and men behave differently: in women, B[a]P-induced C3++ MN were significantly increased (p<0.01), while in men those C3++ MN were non-significantly increased (p<0.1). However, direct comparison of the proportions of C+ and C3++ MN showed no significant differences between the sexes.

Correlations between biomarkers

Since in our study most biomarkers (B[a]P-DNA adducts, mean SCEs per cell, % HFC, MN and CA frequencies) were significantly correlated with B[a]P exposure (see Pearson’s correlation coefficients, Table 4), partial correlation coefficients were calculated, controlling for B[a]P exposure and sex. B[a]P-DNA adducts showed significant partial correlations with both SCEs indicators (mean SCEs per cell and % HFC – p=0.008 and p=0.002, respectively). Interestingly, % HFC also
Fortin F (2015) Biomarkers of genotoxicity measured in human lymphocytes exposed to benzo[a]pyrene: Aneugenic effect, and involvement of multiple primary DNA lesions.

Table 5. B[a]P-DNA adducts measured with the BPDE-DNA CIA in human lymphocytes drawn from 12 subjects, in vitro exposed to benzo[a]pyrene. Results are expressed as adducts per 10⁶ nucleotides.

| Identifier | B[a]P concentrations (µM) | 0 | 0.4 | 4 | 20 |
|------------|--------------------------|---|-----|---|----|
| F1*        | nd                       | 2316 | 17291 | 16881 |
| F2         | nd                       | 5030 | 7093 | 1761 |
| F3         | nd                       | 6757 | 13114 | 12934 |
| F4         | nd                       | 7443 | 23105 | 4166 |
| F5         | nd                       | 11501 | 12740 | 40383 |
| F6         | nd                       | 3132 | 13907 | 7419 |
| F7         | nd                       | 3279 | 5049 | 2054 |
| F8         | nd                       | 3782 | 19307 | 12581 |
| F11        | nd                       | 10171 | 13357 | 12764 |
| F12        | nd                       | 4666 | 15154 | 17458 |
| F13        | 83                       | 3319 | 9159 | 3811 |
| F14        | 11                       | 8402 | 7677 | 2143 |
| F15        | 19                       | 5500 | na   | 3957 |
| M1†        | 98                       | 15699 | 14045 | 5894 |
| M2         | nd                       | 9200 | 19615 | 9203 |
| M3         | nd                       | 2511 | 13727 | 24574 |
| M4         | nd                       | 13077 | 31323 | 38033 |
| M5†         | nd                       | 6315 | 25796 | 32880 |
| M6         | nd                       | 6458 | 18357 | 13357 |
| M7         | nd                       | 17542 | 13978 | 8481 |
| M8         | 55                       | 1099 | 1535 | 674 |
| M9         | nd                       | 1490 | 12131 | 3637 |
| M10        | nd                       | 5434 | 11095 | 2226 |
| M11        | nd                       | 2434 | 4830 | 5682 |

nd: not detected
na: not available – not enough DNA in sample
* F: Female subject
† M: Male subject

showed a significant partial correlation with CA frequency (p=0.020) and the number of MN per MNBN (p=0.034). Finally, MN per MNBN and CA frequency showed a significant partial correlation with each other (p=0.011).

Multiple linear regression analysis was performed to identify the explanatory variables affecting our early biomarkers of genotoxicity. B[a]P exposure was the most important variable affecting % HFC, as it explained 44% of its variability (r²=0.441; p=1.2 x 10⁻⁴). Addition of B[a]P-DNA adducts to the regression explained 53% of observed variability in % HFC (r²=0.528; p=8.5 x 10⁻⁵). As for mean SCEs per cell, B[a]P exposure explained 41% of its variability (r²=0.411; p=2.4 x 10⁻⁴). About CA and MN frequencies, the only significant explanatory variable was % HFC, as it explained 34% of CA frequency’s variability (r²=0.335; p=0.001), and 12% of MN frequency’s variability (r²=0.124; p=0.028).

Discussion

Early biomarkers of genotoxicity show different dose-response curves

In our cohort, 76% of unexposed lymphocyte samples had an undetectable level of B[a]P-DNA adducts, as measured by CIA (Table 5). This is in the upper range of reported values for control or exposed populations (13–77%), when adducts are measured with techniques having similar limits of detection [42–44]. This high rate of undetectable samples may be explained, in part, by application of strict selection criteria in our study, which minimizes exposure to PAH. On the other hand, as the blood samples were obtained from our subjects throughout the year, and as the dietary intake of PAH was not controlled, the unexposed samples containing detectable levels of B[a]P-DNA adducts may reflect charbroiled meat consumption or increased air pollution occurring in winter, as observed in Mexico city [45–47].

Following B[a]P exposure, B[a]P-DNA adducts measurements showed a progressive increase over the control values for the first two B[a]P concentrations (Table 2), and at [B[a]P]=20 µM, B[a]P-DNA adducts significantly decreased (p<0.05). This dose-response curve has an inverted U-shape also observed with CAs and MN. Indeed, significant induction of CAs and MN were observed at all B[a]P concentrations tested (Table 2). As for the B[a]P-DNA adducts, a significant decrease in MN frequency (p<0.05), and a moderate (non-significant – p<0.1) decrease in CA frequency were observed after exposure to [B[a]P]=40 µM, when compared to [B[a]P]=20 µM. Similar observations were reported in the literature, for both in vitro studies and population studies. Indeed, less DNA adducts were observed in human lung fibroblasts exposed to 1 µM B[a]P, when compared to 0.25 µM [48]. Also, higher exposed

Table 6. DNA single-strand breaks quantified in human lymphocytes drawn from 10 subjects, in vitro exposed to benzo[a]pyrene. Results expressed as mean Immunogold particles (IGP) per µm² of chromatin ± SEM, and as means normalised to the negative control ([B[a]P]=0 µM).

| Identifier | Indicator | 0 | 0.4 | 4 | 20 | 40 |
|------------|----------|---|-----|---|----|---|
| F1†        | IGP/ µm² | 93 ± 3.7 | 164 ± 8.1† | 366 ± 12.0† | na | 191 ± 5.4† |
| Normalised | mean     | 1.00 | 1.76 | 3.92 | 205 |
| F4/        | IGP/ µm² | 681 ± 31.6 | 634 ± 26.7 | 466 ± 35.0† | 502 ± 47.5† | na |
| Normalised | mean     | 1.00 | 0.93 | 0.68 | 0.74 |
| F5/        | IGP/ µm² | 233 ± 6.7 | 438 ± 19.8| 358 ± 11.9‡ | 362 ± 8.0‡ | na |
| Normalised | mean     | 1.00 | 1.88 | 1.54 | 1.56 |
| F11        | IGP/ µm² | 312 ± 17.1 | 291 ± 13.7 | 260 ± 15.4 | na | 361 ± 13.0 |
| Normalised | mean     | 1.00 | 0.93 | 0.83 | 1.16 |
| F12        | IGP/ µm² | 52 ± 1.6 | 85 ± 3.2‡ | 39 ± 1.2‡ | 81 ± 3.0‡ | na |
| Normalised | mean     | 1.00 | 1.64 | 0.75 | 1.57 |
| M1†        | IGP/ µm² | 278 ± 13.0 | 306 ± 10.2 | 576 ± 16.9‡ | na | 508 ± 41.9‡ |
| Normalised | mean     | 1.00 | 1.10 | 2.07 | 1.83 |
| M2         | IGP/ µm² | 560 ± 32.2 | 563 ± 23.2 | 538 ± 16.3 | 323 ± 23.9‡ | na |
| Normalised | mean     | 1.00 | 1.01 | 0.96 | 0.58 |
| M3         | IGP/ µm² | 75 ± 7.2 | 86 ± 4.9 | 100 ± 5.4 | 37 ± 1.6† | na |
| Normalised | mean     | 1.00 | 1.14 | 1.33 | 0.49 |
| M4         | IGP/ µm² | 276 ± 13.8 | 231 ± 12.6 | 451 ± 15.8‡ | 577 ± 19.3‡ | na |
| Normalised | mean     | 1.00 | 0.83 | 1.63 | 2.72 |
| M11        | IGP/ µm² | 165 ± 11.4 | 415 ± 24.9‡ | 514 ± 24.5‡ | na | 307 ± 17.8‡ |
| Normalised | mean     | 1.00 | 2.51 | 3.11 | 1.85 |

na: not available – chromosome preparation not available
* F: Female subject
† M: Male subject
p-values are presented for negative control vs B[a]P exposure; One-way ANOVA followed by Bonferroni or Tamhane’s T2 post-hoc test.
†† p<0.01   ‡‡ p<0.001
workers of a coke oven plant presented less MN than medium exposed workers [49]. Furthermore, significantly less bulky DNA adducts and less MN were observed in individuals living in the Ostrava region of Czech republic, when compared to individuals living in Prague, despite higher B[a]P air pollution in the Ostrava region [42,50].

An explanation for this phenomenon is that this decrease could be related to the saturation of metabolic activation enzymes, as suggested by Slikker et al. [51]. A saturation of CYP1A1 enzyme was observed in rat hepatocytes [52], and in breast cancer MCF-7 and HepG2 cells [53], at high benzo[a]pyrene concentrations. In addition, phase II metabolism induction occurs when a sufficient amount of ROS is produced, thereby stabilizing Nrf2 and activating transcription of Nrf2-responsive genes, including aldo-ketoreductases (AKRs), glutathione-s-transferases (GSTs) and other conjugating enzymes [54]. Acting together, those could result in a rapid repair of the DNA damages detected by the COMET assay and, probably, by our EM-ISEL assay, explaining why no significant DNA SSB increase was observed in our setting.

Our results suggest that a third factor, an increased DNA repair rate, could be involved. This is supported by our observation of a linear dose-response curve for mean SCEs per cell and % HFC (Table 2). This could result from the presence of other SCEs-inducing DNA lesions, such as DNA SSBs or 8-OH-dG, which are generated during redox cycling of B[a]P quinones [3]. Those damages were detected in HepG2 cells with the COMET assay, following a 4 h exposure to 2 µM B[a]P [61]. As for human lymphocytes, two studies failed in detecting significant DNA damages with the COMET assay after short-term (1 h and 4 h) B[a]P exposure [18,19], only they were using quiescent lymphocytes, unable to metabolize B[a]P [62]. Even if using cell cycling lymphocytes, our study did not succeed in demonstrating an increased formation of DNA SSBs with the EM-ISEL assay, following B[a]P exposure (Tables 2 and 6). This was also the case for Tao et al. [63], who did not observe significant DNA damages with the COMET assay in bronchoalveolar cells, 24 h post-B[a]P exposure. But when performed immediately or 12 h after B[a]P exposure, the COMET assay did detect a significant DNA damage increase. These results suggest a fast repair of the damages detected by the COMET assay and, probably, by our EM-ISEL assay, explaining why no significant DNA SSB increase was observed in our setting.

B[a]P is a Clastogen and An Aneugen

In our study, exposure to B[a]P induced mainly formation of gaps and chromatid breaks. This is in line with the type of aberrations produced by BPDE in human lymphocytes [64], and classification of B[a]P as a S-dependent agent by Natarajan and Paliti [29]. Moreover, significant increase of all CA types in our study supports classification of B[a]P as a clastogen compound. This classification was realized after review of literature by the GUM group and based on reported significant induction of CA and MN by B[a]P, in various cell systems [30]. In the same way, our study also reports an increased MN frequency (Table 2). On the other hand, we observed significantly increased percentage of cells presenting chromosome losses and gains after B[a]P exposure, maximal at [B[a]P]=20 µM (p<0.001 – Table 2). More, using FISH-coupled MN assay, we found that exposure to [B[a]P]=20 µM induced most frequently formation of MN containing one or more centromeres (C+ MN – group: 77.1% compared to 68.5%; p<0.01 – Table 3). Percentage of C+ MN observed in our study in the control condition (68.5%) is in the range of values reported in literature, which shows a quite high variation: from 33% C+ MN in Decorlier et al. [65], up to 77% in farmarcoi et al. [33]. Intermediate values, such as 45% C+ MN in the control subjects of Vlachodimitropoulos et al. [66] or 65% in Cakmak Demircigil et al. [67] are also reported.

Table 7. Percentage of High Frequency SCE Cells (HFC) in human lymphocytes drawn from 16 subjects, in vitro exposed to benzo[a]pyrene. The 95th percentile of the SCE distribution was determined individually, for each subject, after analysis of their own unexposed cultures. Fifty metaphases per condition (two cultures per condition) were analyzed for all subjects.

| Identifier | 95th percentile SCE distribution (negative control) | HFC cells (%) | B[a]P (µM) |
|------------|--------------------------------------------------|---------------|------------|
|            |                                                   | 0             | 0.4        | 4          | 20         |
| F1         | 13.9 SCE                                          | 4             | 10         | 5          | 44         |
| F2         | 12 SCE                                            | 6             | 50         | 82         | 98         |
| F6         | 11 SCE                                            | 6             | 8          | 62         | 86         |
| F7         | 13 SCE                                            | 10            | 36         | 75         | na         |
| F11        | 13 SCE                                            | 6             | 16         | 32         | 44         |
| F12        | 14 SCE                                            | 6             | 22         | 64         | 64         |
| F13        | 13.5 SCE                                          | 4             | 8          | 66         | 68         |
| F14        | 14.5 SCE                                          | 4             | 52         | 64         | 76         |
| M1         | 20.5 SCE                                          | 4             | 2          | 28         | 38         |
| M2         | 14.9 SCE                                          | 4             | 10         | 60         | 62         |
| M3         | 12.5 SCE                                          | 4             | 26         | 58         | 62         |
| M4         | 11.9 SCE                                          | 4             | 20         | 64         | 64         |
| M5         | 12.5 SCE                                          | 4             | 36         | 80         | 82         |
| M8         | 13 SCE                                            | 8             | 36         | 70         | 84         |
| M11        | 13.9 SCE                                          | 4             | 40         | 58         | 70         |
| M12        | 10.9 SCE                                          | 4             | 42         | 52         | 60         |

na: not available – chromosome preparation not available

* F: Female subject
† M: Male subject

by insertion of different BPDE diastereoisomer adducts in DNA [58]. Using an in vitro repair assay with human lymphoblastoid cell extracts, Custer et al. [59] showed that trans-(±)-anti-BPDE-dG adducts were the less efficiently repaired of all trans adducts. Given that these adducts would be the major ones in blood lymphocytes (as in bronchoalveolar cells), and as they are not efficiently repaired by NER, we can postulate that these adducts could still be present in DNA when replication forks progress. Presence of these unmodified adducts would stall replication forks during the next cell cycle turn, thereby engaging HR repair to a greater extent, and leading to production of an increased number of SCEs. Conversely, if NER repair efficiency was increased, fewer DNA adducts would persist, HR repair would be less engaged and fewer SCEs would be observed. It is actually the case in smoking individuals having an increased NER repair efficiency, where decreased mean SCEs were observed [60].

As we report a significant decrease in B[a]P-DNA adduct level at [B[a]P]=20 µM, a decrease in SCE formation would be expected at this concentration. Clearly, this is not the case here (Table 2). This could result from the presence of other SCEs-inducing DNA lesions, such as DNA SSBs or 8-OH-dG, which are generated during redox cycling of B[a]P quinones [3]. Those damages were detected in HepG2 cells with the COMET assay, following a 4 h exposure to 2 µM B[a]P [61]. As for human lymphocytes, two studies failed in detecting significant DNA damages with the COMET assay after short-term (1 h and 4 h) B[a]P exposure [18,19], only they were using quiescent lymphocytes, unable to metabolize B[a]P [62]. Even if using cell cycling lymphocytes, our study did not succeed in demonstrating an increased formation of DNA SSBs with the EM-ISEL assay, following B[a]P exposure (Tables 2 and 6). This was also the case for Tao et al. [63], who did not observe significant DNA damages with the COMET assay in bronchoalveolar cells, 24 h post-B[a]P exposure. But when performed immediately or 12 h after B[a]P exposure, the COMET assay did detect a significant DNA damage increase. These results suggest a fast repair of the damages detected by the COMET assay and, probably, by our EM-ISEL assay, explaining why no significant DNA SSB increase was observed in our setting.

\[ \text{B[a]}P \text{ is a Clastogen and an Aneugen} \]
We created two new categories to further refine our analysis: C_{1-2}+ and C_{3+} + MN. Those permitted distinction between events possibly involving only one chromosome or its two chromatids (C_{1-2}+ MN), and events necessarily involving 2 or more chromosomes (C_{3+} + MN). This new categorization revealed that, in our study, C_{3+} MN generation after B[a]P exposure contained 3 or more centromeres in a significantly increased proportion, compared to the negative control (group: 78.3% compared to 66.5%; p<0.01 – Table 3). This suggests that B[a]P also has an aneugenic effect on human lymphocytes, and this effect is observed similarly in men and in women (Table 2). As most MN produced by B[a]P contain three or more centromeres in our study, this could be related to an impairment at the centromere level [68]. Indeed, Shimamura et al. [69] found that BPDE exposure of a p53-deficient lung cancer cell line induced an excessive centromere duplication following a prolonged S-phase arrest, and subsequently, chromosome instability appearance. On the other hand, our results also support the findings of Matsuoka et al. [70] on a V79-MZ hamster cell line. They found that in B[a]P-treated cells (1.25 to 10 µM for 24 h), distribution of chromosome number ranged from a diploid to a tetraploid number. Further analyses showed presence of spindle disturbances (incomplete spindles, multipolar spindles and lagging chromosomes) immediately after B[a]P exposure, as well as appearance of hyperdiploidy in V79-MZ cells cultured up to five months after exposure [71]. Interestingly, these cells contain no detectable aryl hydrocarbon hydroxylase activity, suggesting a direct action of unmetabolized B[a]P on cellular components, probably on the mitotic spindle, as observed with classic aneugenic agents such as colchicine and vincristine [72].

Correlations between B[a]P exposure, B[a]P-DNA adducts and early biomarkers of genotoxicity

In our study, early biomarker levels (B[a]P-DNA adducts, SCEs, % HFC, MN and CA frequencies) correlated significantly with B[a]P exposure (Table 4). Those correlations were not established in previous published in vitro studies using human lymphocytes. Nevertheless, correlations were analyzed in occupational studies (e.g. non experimental designs) with contradictory results. Indeed, mean SCEs, MN frequency and/or CA frequency were associated with PAH exposure in coke oven workers and coal exposed workers in many studies [6,8,9,73], while no association was found in other reports [7,49]. The same contradictory situation was reported for DNA adducts and PAH exposure, as reviewed by Brandt and Watson [74].

Investigation of relations existing between early biomarkers measured in our study revealed that B[a]P-DNA adducts correlated significantly with the two SCE indicators (mean SCEs per cell and % HFC; p=0.008 and p=0.002, respectively – Table 4), when controlling for B[a]P exposure and sex. In literature, significant positive correlations between SCEs and B[a]P-induced or bulky DNA-adducts were reported only in one in vitro study conducted on human lymphocytes of two subjects [71], and in one cohort study exposed to environmental pollution in Upper Silesia (Poland) [75]. However, in coke oven workers, no significant correlations were observed between SCEs or % HFC, and bulky DNA-adducts [14,76].

In our study, multiple linear regression analysis showed that a large part of the variability present when analysing SCE indicators (mean SCEs per cell and % HFC) can be explained by B[a]P exposure and by B[a]P-DNA adducts. This analysis also showed that % HFC is the only variable significantly affecting CA and MN frequencies, providing a link between SCEs, CAs and MN. Furthermore, we report a significant correlation existing between CA frequency and MN per MNBN (r=0.351, p=0.011; Table 4), likely related to the role of chromosome breaks in MN formation [32]. On the other hand, in addition to DNA adducts, other DNA lesions caused by B[a]P exposure, such as DSBs and 8-OH-dG, are also known to lead to SCE formation [56]. While DSBs are responsible for CA and MN formation [77], importance of 8-OH-dG in MN formation should not be excluded, as MN and 8-OH-dG increases were significantly associated with PAH exposure in coke oven workers [9]. Also, as B[a]P quinones were detected in B[a]P-treated human lymphocytes [16], 8-OH-dG formation is almost certainly present in this cell type. In our study, because % HFC’s variability is linked to B[a]P-DNA adducts, CA frequency and MN frequency (when excluding effect of B[a]P exposure and sex), this gives support to a role of B[a]P-DNA adducts, DNA DSBs and 8-OH-dG, as DNA primary lesions, in the production of cells containing a high number of SCEs. These factors could explain why % HFC is considered a more sensible genotoxic endpoint than mean SCEs per cells [78]. At last, heritability has a major role in MN and CA formation, explaining up to 70% of interindividual variation [79,80], while it explains only 30% of interindividual variation in SCEs [81]. Thus, it is not surprising that, in our study, an environmental factor such as B[a]P exposure, cannot significantly explain the observed variability in CA and MN frequencies. Only % HFC, which reflects action of both environmental and genetic factors, significantly explains a small part of CA and MN frequencies (34% and 12%, respectively).

Men could be more sensitive to genotoxic effects caused by low B[a]P exposure

In our cohort, sex differences were investigated by direct comparison of all early biomarker levels (B[a]P-DNA adducts, DNA SSBs, SCE, CA and MN), between men and women. Following B[a]P exposure of blood lymphocytes, statistical analysis did not reveal significant sex differences (bilateral Student’s t-test for independent samples – Table 2), even if these levels were increased in women. On the other hand, when analyzing the dose-response curves observed in women and men separately, an increased sensitivity of men showed up, especially to our lower B[a]P concentrations. Taking into account B[a]P-DNA adducts, men presented more adducts than women in all B[a]P-exposure conditions, even though this difference did not reach statistical significance (Table 3). Also, men presented a significant increase in the following biomarkers at [B[a]P]=0.4 µM: mean SCEs, % HFC, CA frequency and MN frequency (Table 2).

Sex differences in DNA damages have not been reported frequently and could be related to specific exposures. In individuals exposed to air pollution, a significantly increased bulky DNA adduct level in lymphocytes was reported only in males [82]. On the other hand, in Upper Silesia (Poland), significantly increased blood DNA adduct level, mean SCEs and MN frequency were found in girls, but not in boys, in response to a complex environmental exposure [75]. Sex differences in genotoxic response to B[a]P or PAHs could be associated with different metabolic capacities, specific for a particular cell type. In fact, women have a significantly lower baseline CYP1A1 activity in blood cells, and this activity is less inducible by 3-methylcholanthrene, when compared to men [83]. This could explain the lower B[a]P-DNA adducts level observed in women of our cohort. On the contrary, significantly more bulky DNA adducts are found in lung tissues taken from non-smoking females, when compared with non-smoking male tissues [84]. Furthermore, lung adenocarcinoma cell lines of female origin have significantly more B[a]P-induced DNA adducts, increased basal and induced CYP1A1 levels, and increased CYP1A1 activity [85]. Together,
these findings support the concept that sex differences could exist, and may be related to specific exposures and cell types.

In summary, our study shows that, when measuring early biomarkers (B[a]P-DNA adducts, MN and CAs), an inverted-U dose-response curve is present in human lymphocytes exposed to low B[a]P concentrations. This particular dose-response curve could be caused by the coordinate action of three pathways, namely a saturation in metabolic activation enzyme activity, the induction of phase II metabolic enzymes, and an increased DNA repair rate in high B[a]P exposure conditions, as observed in some population studies. On the other hand, as SCE test gave a linear dose-response curve, it suggests that some DNA lesions, such as unrepaired DNA adducts or 8-OH-dG, engaged HR repair to a greater extent at high B[a]P concentrations. As unrepaired adducts could persist, a kinetic study evaluating formation and repair rates of B[a]P-DNA adducts in human lymphocytes could be interesting, using similar and lower B[a]P concentrations. Also, our multiple regression analysis independently linked % HFC’s variability to three other biomarkers: B[a]P-DNA adducts, CA frequency and MN frequency, when controlling for B[a]P exposure and sex. Since % HFC is the only variable significantly affecting CA and MN frequency, and since CA and MN are formed through a common DNA lesion, namely DNA DSBs, this suggests a role for DNA DSBs, and probably for 8-OH-dG, in the production of cells containing a high number of SCEs.

Additionally, clastogenic activity of B[a]P in normal (untransformed) human cells was confirmed by our results. And, most importantly, aneugenic activity of B[a]P on human lymphocytes was suggested by the presence of metaphases presenting chromosome losses and gains after B[a]P exposure. This was confirmed by FISH-coupled MN assay showing that most B[a]P-induced MN contained three or more centromeres, also suggesting an aneugenic effect. Use of increased proportion of C> MN and/or C< + MN as biomarkers of aneugenic activity must be validated. The work recently published by Speit et al. on formaldehyde [72] describes an interesting approach to in vitro characterization of potential aneugen compounds integrating FISH-coupled MN assay to more classical assays. This integrative approach could be interesting to investigate further the aneugenic activity of B[a]P on human cells. Our results, together with karyotypic changes [70], spindle disturbances [71] and centrosome duplication [69], provide additional evidences that B[a]P could play a role in the progression step of carcinogenesis. Involvement of B[a]P and other PAH during cancer progression needs to be confirmed in studies involving humans environmentally or occupationally exposed to PAH, particularly using FISH-coupled MN assay. This might be of particular importance given that exposure to B[a]P and other PAHs (smoking, environmental and occupational exposure) are chronic and may last for decades.

Funding

This work was supported by the département de pathologie du Centre Hospitalier Universitaire Sainte-Justine [to N.L.], the Fonds de la Recherche en Santé du Québec (FRSQ) [to F.F.], FRSQ’s Environmental Health Research Network [to P.A.] and the Fondation de l’Hôpital Sainte-Justine/Fondation des Étoiles [to F.F.].

Acknowledgment

Special thanks Miriam C. Poirier from the Center for Cancer Research, National Institutes of Health (Bethesda, MD, USA) who provided us with the PAH-DNA antisera. The authors would also like to thank all subjects for their cooperation while participating in this study.

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