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Analysis of DNA Damage Biomarkers in Human Leukocytes by PAHs Exposure

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Authors' contributions

This work was carried out in collaboration between all authors. Author RUH designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors MLVB and EUV managed the analyses of the study. Author ERG managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To study the potential genotoxicity of two polynuclear aromatic hydrocarbons (PAHs), exposed cultured human leukocytes in vitro using two types of biomarkers genotoxicity: DNA strand breaks (DNA-SB) and adduct formation (DNA-PAHs).
Study Design: Human leukocytes were exposed to toxic cultures with different concentrations of anthracene (ANT), phenanthrene (PHE) and benzo(a)pyrene (B(a)P) for 24 hours. Four toxic test groups, PAHs, control group, analytic blank group and standard fluorescence group were considered.
Place and Duration of Study: Laboratorio de Citopatología Ambiental, of Departamento de Morfología at Escuela Nacional de Ciencias Biológicas, IPN; between february 2016 and july 2018.

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Methodology: Human leukocytes cells were isolated and cell viability was previously verified by Trypan dye exclusion test. Firstly, the lethal concentrations with neutral red (NR50) assay for each one PAHs was obtained. Then sublethal concentrations range of these toxics for both biomarkers were used. In case of DNA fragmentation, a fluorochrome was used to mark DNA strandbreaks and isolation with alkaline solution finally determined with fluorescence spectroscopy. To test the formation of DNA-PAHs adducts, first of all they were isolated with a solvent system with polarity gradient and finally determined with fluorescence spectroscopy.
Results: PAHs with 3 aromatic rings showed lethal cytotoxicity, lower in case of the B(a)P with 5 aromatic rings. These results are in contrast with previously reported observations, in which non-adduct with DNA and DNA strandbreaks formation were detected with Anthracene and Phenanthrene while DNA adduction formation and DNA strandbreaks were produced in case of B(a)P.
Conclusion: Biomarkers may be used as suitable discriminants of genotoxic agents as well as of environmental pollutants with genotoxic potential and for application in studies of environmental risk assessment and in hazardous waste evaluation. The application of both genotoxic biomarkers, DNA strand breaks and production of adducts DNA-PAHs, as genotoxicity assays are quickly and accurate techniques for determining the carcinogenic potential of environmental samples.

Keywords: Genotoxicity biomarkers; DNA fragmentation; DNA adducts; PAHs; B(a)P; anthracene; phenanthrene.

ABBREVIATIONS

Polynuclear Aromatic Hydrocarbons (PAHs), DNA Strand Breaks (DNA-SB) Anthracene (ANT), Phenanthrene (PHE), Benzo(a)pyrene (B(a)P).

1. INTRODUCTION

The polynuclear aromatic hydrocarbons (PAHs) can be found almost everywhere, in the air, land and water, from natural sources or anthropogenic. The contribution of natural, such as forest fires and volcanoes, is minimal compared with the emissions caused by humans. The combustion of fossil fuels is the main source of emission of PAHs. Other emissions come from the combustion of waste and wood, as well as discharges of crude or refined petroleum containing PAHs in itself. These compounds are also present in tobacco smoke and grilled, smoked foods and fried foods [1].

The main source of PAHs in the air is coal tar, formed by pyrolysis of coal in factories of gas and coke, which produce emissions of fumes from heated pitch. PAHs are formed by pyrolysis or incomplete combustion of organic matter that contains carbon and hydrogen. At high temperatures, the pyrolysis of organic compounds produces fragments of molecules and radicals that combined give rise to PAHs. The composition of the pyrosynthetic products depends on fuel, temperature and time spent at high temperatures. Fuels that are PAHs source are methane, other hydrocarbons, carbohydrates, lignins, peptides, etc. [2].

Chemically, PAHs react by addition or substitution of hydrogen when there is saturation, keeping the system of rings. Most of the PAHs, suffer photooxidation, this being a way to remove them from the atmosphere. The most frequent photo-oxidation reaction is the formation of endoperoxides, by which they can be turned to Quinones. PAHs react rapidly with oxides of nitrogen or nitric acid. For example, anthracene can be oxidized to anthraquinone by the action of nitric acid and nitrogen dioxide or give a nitrogen derivative by a nitrogen dioxide replacement reaction [2].

The World Health Organization (WHO) determined of 0.2 mg/m^3 as limit of occupational exposure to B(a)P. Additionally, WHO guidelines for drinking water quality in 1993 concluded sufficient data for PAHs other than for Benzo(a)pyrene, for which the guideline value was calculated to be 0.7 µg/L. In Mexico, the limit for PAHs particles has been set at an average time of 24 hrs. of exposure 0.3 mg/m^3 (IARC, 2010). In Europe, the emission of PAHs has been recognized as a serious public health problem; Stockholm emits high concentrations of PAHs with an averaging concentration of 100 to 200 ng / m^3. 14 PAHs that are measured as air pollutants B(a)P concentration is in the range of 0.4 -0.2 ng / m3 while Phenanthrene (PHE) is 10 times higher [3].

The International Agency for research on Cancer (IARC), established in its 1983 report that 11 polycyclic aromatic hydrocarbon show a sufficient carcinogenesis evidence in experimental animals, also based on epidemiological studies which showed a close relationship with the increase in the incidence of cancer in workers exposed to PAHs [4].

Anthracene (ANT), also known as para-naftalene, is a polycyclic aromatic hydrocarbon that, according to the [5] U.S. Environmental Protection Agency (US-EPA), it is not classified as carcinogenic for human and designated by the letter (D). However, there is no data in humans and the information in the case of animals is inadequate. B(a)P according to the information provided by the US-EPA [6], is classified as a probable carcinogen for humans (B2).

The exposure of an organism to a genotoxic chemical substance can induce a cascade of genetic events, appearing initially as alterations in the structure of DNA. Damage is then processed, subsequently expressed in mutation and finally the products evolve in a cancerous process.

In the case of PAHs the main studied mechanism of its genotoxicity is through the formation of adducts between the DNA molecule and the metabolites of PAHs. Another no less important mechanism is the breaking of the double-stranded DNA molecule into fragments, induced by oxidative damage caused by PAHs, which may result in DNA denaturation [6]. The formation of DNA fragments can have a major impact on the processes of mutagenesis and carcinogenesis. DNA adducts are considered sensitive biomarkers of PAHs exposure and these effects are considered to be a cumulative index of current and past exposure to genotoxic compounds [7].

Carcinogenicity is believed to be due to many PAHs. The mechanism by which individual PAHs initiate chemical carcinogenesis has been extensively investigated [8]. PAHs are metabolically activated as electrophilic reactive that covalently modify cellular DNA forming DNA-PAHs adducts. It is generally accepted that chemical DNA adducts formation in organs susceptible to PAHs carcinogenesis is the first critical event in a multistep process that leads to cancer induction.

PAHs have received public attention by the fact that many of these compounds are genotoxic in humans. B(a)P is a typical representative of PAHs carcinogen product, and this substance is metabolically activated prior to the formation of DNA adducts. Although a series of epoxy and epoxy diol metabolites formed from B(a)P are mutagenic, 7-, 8-diol - 9, 10-epoxy is believed to be the ultimate carcinogen, at least for mammals [9]. Metabolism of B(a)P differs among species, and DNA-B(a)P adducts are a fingerprint of this.

The adducts are chemical compounds covalently linked to large structures such as DNA and other macromolecules including hemoglobin and other proteins. Changes in the DNA molecule are produced by the majority of the resulting metabolites of PAHs, which epoxides with nearby diols are able to attack the amino groups of guanine and adenine [7]. The metabolic pathway followed by PAHs (taking B(a)P as an example), from their uptake to the formation of adducts is described by a process beginning once PAHs enter into the cell and form various epoxides by oxidative action of the mono oxygenase, microsomal cytochrome-dependent p450 (CYP1A).

These epoxies can rearrange spontaneously in phenols, hydrolyzed to hydrodiols by the epoxido-hydrolases or covalently join glutathione (spontaneously or catalyzed by glutathione-S-transferase). Some phenols are oxidized to Quinones while others produce secondary epoxides (di-hidrodiolepoxidos), which are more reactive to form DNA adducts [10,11,12].

2. MATERIALS AND METHODS

2.1 Chemicals

Dimethyl Sulphoxide (DMSO), polyvinylpyrrolidone (PVP), coproporphyrin I (5 µg/vial) and tetrazolium salt (NBT) (purity 98%) were acquired from Sigma Chemical Company (St. Louis, MO., USA). Anthracene (ANT) (97% pure), Phenanthrene (PHE) (95%) and B(a)P (98% pure) were purchased form the Kanto Chemical Inc. (Tokyo, Japan). Neutral red (75% pure) and trypan blue dyes (80% pure) were obtained from Merck (New Jersey, USA). Culture media RPMI-1640 and the antibiotic mixture were acquired from In Vitro (Mexico City, Mexico). All other chemicals utilized in the investigation were analytical grade.

The PAHs solutions were prepared in dimethyl sulfoxide (DMSO) 0.5% with culture medium RPMI-1640.5xE4 cells were used in each test. The concentrations of PHAs tested to determine

the lethal cytotoxicity were 0.02 to 0.08 µM for ANT, 0.02 to 0.08 µM for PHEN and 0.5 to 4.0 µM for BaP, including a negative control with RPMI-1640 medium and DMSO witness. All solutions were sterilized with a 0.22 µm membrane filter.

2.2 Isolation of the Leukocytes Fraction

Fresh blood samples were obtained from a blood bank, located in Mexico City, under the terms and conditions on Mexico's Ministry of Health for the utilization of blood samples for research purposes. Plasma, platelets and erythrocytes from blood samples were separated by 30-minute centrifugation at 700 rpm using a density gradient with Poly Vinyl Pirrolidone 1% solution and after washing with Ammonium Chloride 0.8% again centrifugated at 700 rpm. The leukocyte fraction was suspended in RPMI-1640 culture media at 1.0-2.0E6 cells/mL. Leukocytes viability was determined by the Trypan blue method according to Del Raso [13], using cell viability > 90%, with 24 h period for acclimatization.

2.3 Lethal Cytotoxicity Assay to ANT, PHE and BaP

Subsequently the exposure for 24 hours at 37°C to PHAs, a cytotoxicity test was performed with the supravital dye Neutral Red (NR) [14]. NR is absorbed by the lysosomes of living cells. Then dye was extracted and quantified by visible spectrophotometry by determining the light absorption at 540 nm. The fifty percent lethal cytotoxicity concentration (LC50) was calculate by linear regression. Sublethal concentrations, minors to the LC50, were used in the assays to test DNA strand breaks and formations of adducts DNA-PAHs.

2.4 Determination of DNA Fragmentation

The technique of fluorescence analysis of DNA unwinding (FADU) [15] was used for the quantification of strandbreaks in the DNA chain. This technique is based on the time-dependent alkaline denaturation of DNA under conditions of moderate denaturation using NaOH 0.1 M, pH= 14, since in severe alkaline conditions DNA is unwound completely. In moderate alkaline conditions DNA denaturation is a time-dependently process, that has to be stopped after a certain period of time by neutralization with HCl 0.1 M. Samples are sonicates to 100 W, 15 s, then bisbenzamide 1.25 µM is added and finally the fluorescence is determinated by spectrofluorometry (Luminescence Spectrometer, model LS 50, Perkin-Elmer) [16]. Emission wavelength was set to 450 nm and excitation wavelength to 355 nm Results are expressed as relative fluorescence intensity (Fr).

2.5 Determination of Adducts DNA-PAHs

Cell samples were withdrawn from the culture medium, pelleted and re-suspended in 1 mL of PBS. Extraction of DNA was realized with a volume of ethyl acetate in agitation in vortex for 3 min, leaving to stand at room temperature for 5 min. For the precipitation of the DNA were added two volumes of isopropanol by swirling Vortex for 3 min, steeping the mixture at 4°C for 5 min. It was centrifuged at 1500 rpm for 10 min. The solvent was removed and washed with a volume of ethyl alcohol by swirling Vortex 3 minutes. It was finally re-suspended in a volume of water [17].

DNA-PAHs adducts were measured by spectrofluorometry (Luminescence Spectrometer, Perkin-Elmer, model LS 50) [15]. Adducts formation were estimated by fluorescence intensity at λex = 417 nm using an excitation wavelength of λex =300 nm and the extent of adducts formation was calculated as the fluorescence relative intensity obtained after correction with control and blank.

2.6 Statistical Analysis

All tests were performed by triplicate. The values are means ± standard deviation (SD). ANOVA analysis was realized to evaluate the difference between PAHs concentration groups for the biomarkers genotoxicity responses. Linear regression analysis was used to determine the relation of concentrations of each PAHs with DNA strand breaks and DNA-PAHs adducts formation. All analyses were performed using the software SPSS® v.22 [18] with $p< 0.05$ considered significant.

3. RESULTS

Lethal cytotoxicity test to ANT yielded a LC50 = 0,352 µM ($r = 0.94$, $P < 0.01$), a LC50 = 1,521 µM ($r = 0.94$, $P < 0.01$) for PHE and a LC50 = 3.23 µM was obtained in the case of B(a)P ($r = 0.94$; $P<0.001$). The lower LC50 observed for ANT and PHE, indicate that they have more potential lethal cytotoxicity in the conditions of cultivation using leukocyte cells.

The higher cytotoxicity of ANT and PHE in respect to B(a)P can be explained with the chemical properties (lipophilic and low molecular weight)of both ANT and PHE, which allows these compounds to enter the cell and its organelles (mitochondria and nucleus) by passive transport [19].

Table 1 presents the results of DNA strand breaks (DNA-SB) generation as well as DNA-PAH adducts formation by exposure to sublethal concentrations of ANT, PHE and BaP. The results of both kinds of biomarkers assay are expressed as relative fluorescence intensity (Rf).

There were no statistical differences in the levels of DNA fragmentation and formation of DNA-PAHs adducts (Table 1), expressed as relative fluorescence. With exposure to different concentrations of the ANT and PHE, while exposure to B(a)P induced a significant increase ($p < 0.05$) in the production of DNA strandbreaks, as well as in the formation of DNA-B(a)P adducts, at almost all concentrations tested with respect to the negative control.

In the case of the induction of DNA-B(a)P adducts formation, a statistical difference with control was observed ($p < 0.05$) at all concentrations of B(a)P (Fig. 2), with the exception of the lowest one. It is important to note that there is a proportional relationship increasing the DNA response, with respect to the increase in B(a)P concentrations.

In the case of DNA fragmentation, only statistical difference was observed ($p < 0.05$) between the control and the three highest concentrations of B(a)P (Fig. 3). It is important to note that there is an increasing fragmentation in the DNA response, with respect to the increase in B(a)P concentrations.

Table 2 presents the results of the linear regression analysis of both the results of genotoxic biomarkers (DNA strand break and

Table 1. Results of DNA damage biomarkers (DNA strand breaks and induction of DNA-PAHs adducts) at different concentrations (µM) of ANT, PHE and BaP

PAHs compounds	DNA-STRANDBREAK	ADUCCT DNA-PAHs
ANT	**DNA-SB**	**DNA-ANT**
µM	**Rf**	**Rf**
Blank	0.091 ± 0.05	0.083 ± 0.05
Control	0.291 ± 0.05	0.226 ± 0.11
0.01	0.311 ± 0.01	0.288 ± 0.25
0.02	0.329 ± 0.04	0.437 ± 0.1
0.04	0.289 ± 0.05	0.848 ± 0.31
0.06	0.306 ± 0.07	1.204 ± 0.23
0.08	0.379 ± 0.09	2.044 ± 0.51
PHEN	**DNA-SB**	**DNA-PHEN**
µM	**Rf**	**Rf**
Blank	0.087 ± 0.03	0.087 ± 0.05
Control	0.263 ± 0.05	0.331 ± 0.13
0.01	0.327 ± 0.08	0.388 ± 0.25
0.02	0.298 ± 0.04	0.417 ± 0.1
0.04	0.336 ± 0.04	0.448 ± 0.13
0.06	0.367 ± 0.07	0.381 ± 0.17
0.08	0.327 ± 0.09	0.421 ± 0.16
BaP	**DNA-SB**	**DNA-BaP**
µM	**Rf**	**Rf**
Blank	0.075 ± 0.04	0.087 ± 0.15
Control	0.311 ± 0.06	0.378 ± 0.65
0.5	*2.127	*0.865 ± 0.52
1	*4.304	*1.313 ± 0.61
2	*6.822	*2.544 ± 0.33
3	*12.859	*3.613 ± 0.42
4	*23.41	*6.133 ± 0.73

**ANOVA test with statistic difference respect to the control p<0.05.*

DNA-PAHs adducts formation), after exposure to the ANT, PHE and B(a)P. It can be observed a higher coefficient of correlation between the formation of adducts with B(a)P (r = 0.96),with respect to ANT (r = 0.19) and PHE (0.091). A similar situation can be observed for DNA fragmentation and exposure with B(a)P (r = 0.94). This suggests the existence of different toxicity mechanisms for each of the studied PAHs. This difference can be determined by several factors such as the molecular structure as well as the ionization potential. These results provide evidence that chloroperoxidase activity in the leukocytes can mediate binding of PAHs to DNA by one-electron oxidation as showed by Marquez-Rocha et al. [17].

A valid linear relationship ($p < 0.05$ as well as r = 0.96) between PAHs exposure and the genotoxicity biomarkers was obtained only with B(a)P.

Fig. 3 shows the linear relationship between DNA fragmentation and B(a)P concentrations, in which the linear equation is included, whose slope or rate of change implies 5.73 fragmentation units produced by 1 μM unit of B(a)P, and a basal value of 0.37, in the absence of the toxic compound.

Fig. 4 shows the linear relationships between the formation of DNA adducts and the concentrations of B(a)P, which includes the linear equation, whose slope or rate of change implies 1.38 units of adducts produced by 1 unit μM of B(a)P, and a basal value of 0.09, in the absence of toxic compounds.

4. DISCUSSION

We found PAHs with 3 aromatic rings to be acutely cytotoxic while a lower cytotoxicity was detected for PAHs with 5 aromatic rings. These results are in contrast with those observed with the DNA-PAHs adduct production test, in which no adduct formation was detected for ANT, with 3 aromatic rings while B(a)P, with 5 aromatic rings, resulted in adduct formation.

Both DNA strand breaks and DNA-PAH adducts formation analysis has been extensively used to evaluate the toxicity of PAHs. Therefore, employing of DNA-SB and DNA adducts as biomarkers to assess the genotoxicity potential and bioavailability of PAHs and other organic compounds persistent from contaminated soil and water is feasible. The use of DNA-SB and DNA adduct formation to estimate PAHs genotoxicity may provide additional insight into

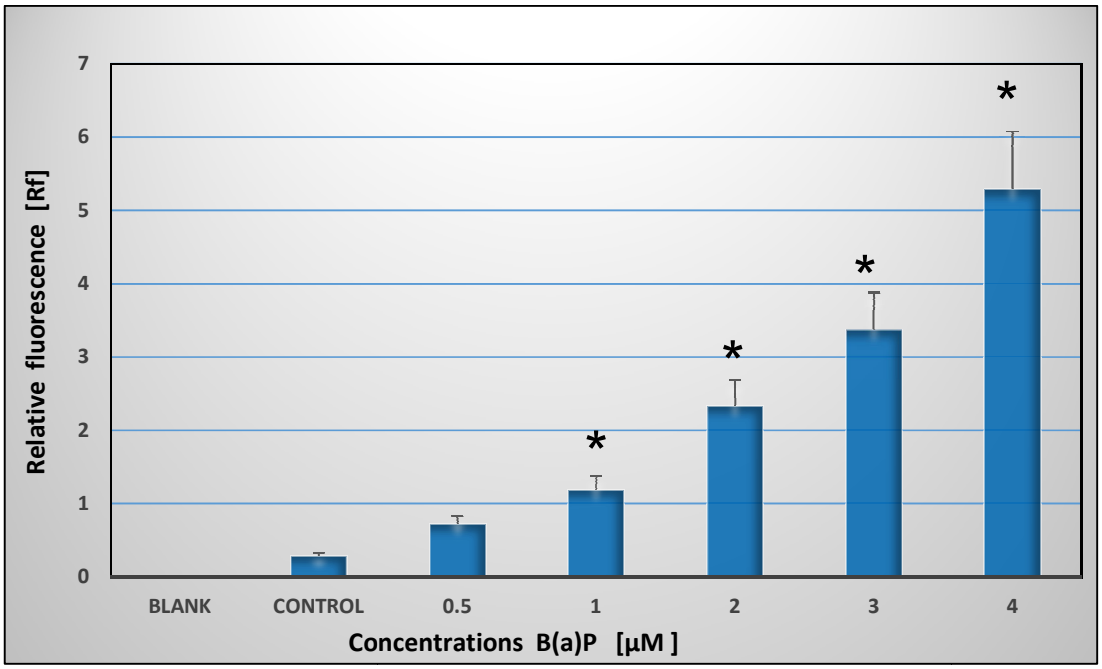

Fig. 1. Formation of adducts with different concentrations of B(a)P. The concentration of B(a)P groups marked with an asterisk, presented statistical difference ($p<0.05$) in comparison at the negative control without toxic. Results are in triplicate, Rf: Relative fluorescence

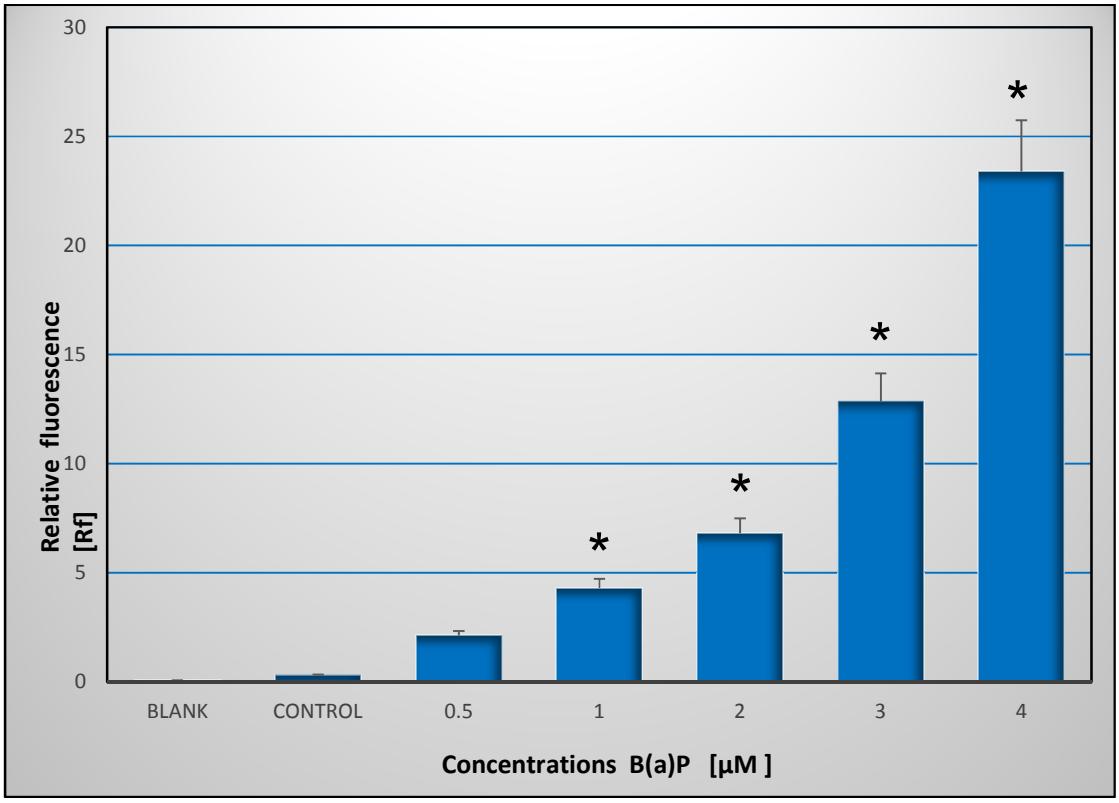

Fig. 2. DNA strand breaks with different concentrations of B(a)P. The concentration of B(a)P groups marked with an asterisk, presented statistical difference($p<0.05$) in comparison at the negative control without toxic. Results are in triplicate, Rf: Relative fluorescence

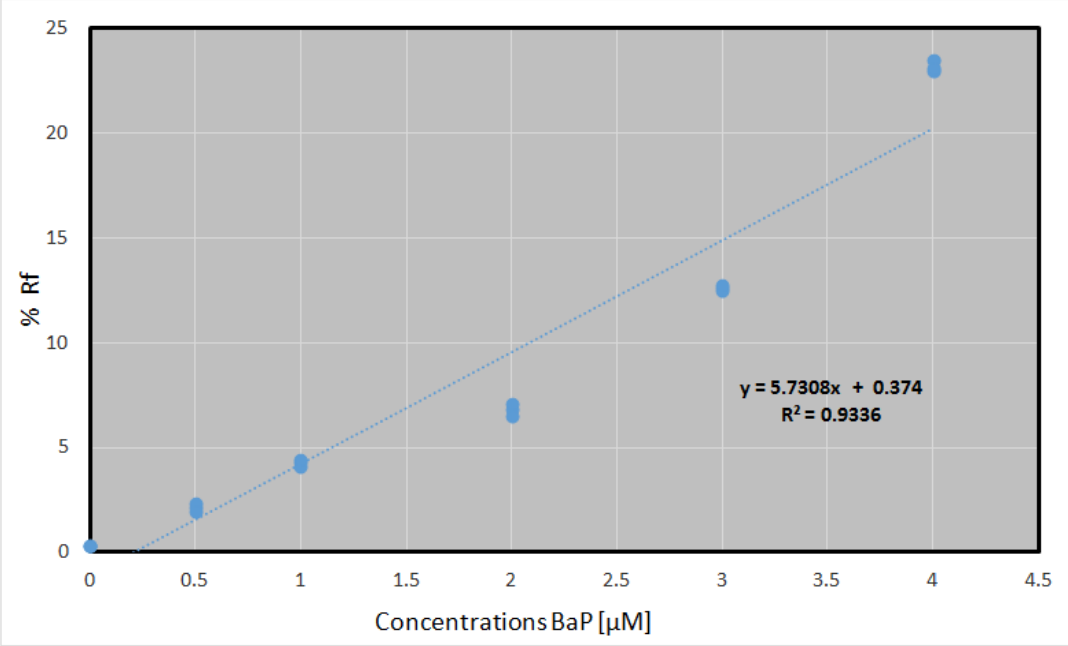

Fig. 3. Linear relationships of DNA strand beaks with increasing B(a)P concentrations.

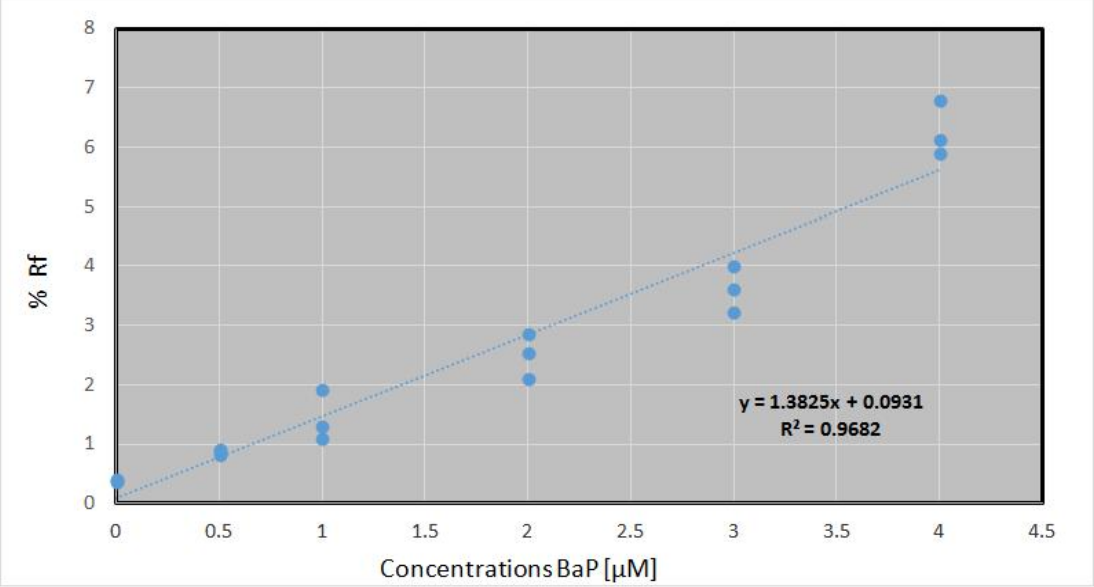

Fig. 4. Linear relationships of the formation of DNA adducts with increasing B(a)P concentrations

Table 2. Parameters of linear regression between PAHs exposure and genotoxicity biomarkers

Genotoxicity biomarker	Anthracene	Phenantrene	B(a)P
DNA strand breaks	m = 0.104	m = 0.091	m=5.76
	b=0.31	b=0.16	b=0.37
	r=0.07	r=0.15	r=0.96
	p>0.05	p>0.05	p<0.05*
DNA-PAHs adducts	m = 0.84	m = 0.34	m=1.35
	b=0.26	b=0.21	b=0.09
	r=0.19	r=0.091	r=0.98
	p>0.05	p>0.05	p<0.05*

**ANOVA test with difference respect to control*

mechanism of action for complex organic mixtures.

The response of the biomarkers of genotoxicity, for the exhibition to Anthracene and Phenantrene, not reflects any relationship ($p > 0.05$). As in the case of the B(a)P with both biomarkers presented a high degree of correlation ($r > 0.95$, $p < 0.01$). The response of them PAHs is consistent with the molecular structure and the grade of reactivity associated to the same, of such compounds, so the Anthracene and Phenantrene not generated a response associated of them biomarkers, while in the B(a)P if is present, this is due high potential of ionization and oxidation associated to the B(a)P. It is important to emphasize that the genotoxic response mechanism is different for these two compounds of the family of PAHs and that can form the basis of the trigger or not the process of carcinogenesis.

It has been reported that in PAHs with three aromatic rings, the acute cytotoxicity has been greater than that which has been detected in PAHs with 5 aromatic rings [20]. These results are in agreement with those observed in the present study with the DNA-PAHs adduction test. The formation of adducts was not present in the case of anthracene and phenanthrene with 3 aromatic rings; while B(a)P with 5 aromatic rings resulted in adducts formation.

The presence of xenobiotic agents activates the NADPH oxidase in polymorphonuclear cells, producing in this reaction the superoxide radical, which is then converted to H_2O_2. The latter induces the activity of myeloperoxidase, an

enzyme that produces hypochlorous acid (HOCl), a potent antibacterial agent in neutrophils [21].

The observed situation suggests that the toxicity mechanism of B(a)P, both at the cytotoxic or genotoxic level, may be associated with the production of reactive oxygen species, which are produced when the B(a)P is oxidized to its quinone form. In the present study, the results of Fabiani et al. [22] show that exposure of monocytes to B(a)P increases genotoxicity compared to control values ($P < 0.001$), while with three-ring aromatic hydrocarbons, such as anthracene, no effect was observed.

Myeloperoxidase is an abundant enzyme in neutrophil leukocytes, with peroxidase activity that depends mainly on the presence of chloride ions, pH, and H_2O_2. The B(a)P is oxidized by the activity of this enzyme at the expense of the reduction of H_2O_2, producing the cationic radical of B(a)P and later the quinone of this compound [17]. It has been suggested that the metabolic activation of PAHs by leukocytes, to reactive intermediates, can be an important step in the increase of superoxide radical production [22] and this in turn in the expression of genotoxic damage.

The DNA strand breaks response produced by the B(a)P exposure showed a high correlation coefficient that is reflected in the fragmentation rate of 5.76 Rf produced by each 0.1μM of B(a)P. In considering the level of basal fragmentation in the leukocytes analyzed it was 0.374% in the absence of B(a)P and the EC50 corresponding to said ratio is 1.07μM B(a)P (Table 2). Not so in the case of ANT and PHE, a situation that can be explained in terms of the bioactivation [23]. These compounds produce reactive derivatives, a fact that is determined by the lower ionization potential of B(a)P (7.12 eV) with respect to anthracene (7.55 eV) and phenanthrene, which favors the oxidation of B(a)P to its cationic radical and later to its quinone derivative.

The presence of DNA strand breaks has been associated with the activity of reactive oxygen species, such as hydrogen peroxide, superoxide and hydroxyl radicals [24]. DNA fragmentation is produced by exposure to H_2O_2 in the range of 25 to 10 μmol/L. Similar to the present study [25] observed that lymphocytes treated with concentrations of 0.6 to 4.8 μM of B(a)P metabolite diolepoxide produced a significant linear increase in DNA fragmentation. In the work done by Johnsen et al. [26] also showed an increase in the proportion of DNA strand fragments in human lymphocytes exposed to 30 μg/ml of a B(a)P derivative for 24 hrs. The derivative is a cyclopenta-B(a)P produced by the metabolism of hepatic microsomes.

In this way, the activation of compounds such as PAHs by the peroxidases present in the polymorphonuclear cells represents an alternative metabolic pathway previously considered [17,27] but that, however, it has not received due attention in the activation of indirect carcinogens or procancerigens. Based on the results, it was determined that anthracene and phenanthrene had a higher LC50 value, with a concentration 9.1 times higher than B(a)P, which coincides with that reported by Bolton et al. [23] and that reflects the higher bioavailability of anthracene and phenanthrene.

These results confirm the clastogenic effect of BaP, related to the production of the superoxide radical, a relationship that may constitute the basis for suggesting a mechanism of oxidative damage to the DNA molecule through the induction of this reactive oxygen species. The metabolism of PAHs is activated by the peroxidase route [28]. The relationship obtained justifies the use of DNA strand breaks as an adequate biomarker of exposure to this genotoxic compound, with an important application in the monitoring of environmental and occupational exposure.

The constant exchange between the oxidized and reduced forms of B(a)P quinone induces the highest production of the superoxide radical with respect to anthracene [20]. Similarly, Borm et al. [29] observed that leukocytes had the capacity to metabolize B(a)P and generate DNA damage by formation of DNA-B(a)P adducts.

In a study in mice exposed to B(a)P there was the highest amount of adducts/μg of DNA, whereas with Chrysene and B(a)A, a smaller amount was induced. In mice exposed to B(a)P there was more than 50 times more DNA adduct / g than Chrysene when the PAHs were administered with a probe, the adduction of the DNA was considerably reduced. B(a)P was the most potent inducer of DNA adducts, so the amount of adducts formed/ μg DNA was almost 30 times lower than when administered by i.p. [30].

Each of the PAHs induced a spectrum of DNA adducts that were similar between species and routes of administration. Little difference was

observed in the number or types of induced adducts, suggesting similar pathways of metabolic activation that are operating in both species [30].

Although the numerical values for the final genotoxic effects called adducts are influenced by the variability between the types of PAHs compounds and their metabolic activation. The absence of trends in the genotoxic biomarkers are evident in our data for both the ANT and PHEN, a fact that determines specific mechanism of genotoxicity associated with the response observed in B(a)P, in which metabolic activation is associated with the production of reactive oxygen species for both types of genotoxic biomarkers [31].

5. CONCLUSIONS

In conclusion, the biomarkers may be used as suitable discriminants of genotoxic agents as well as of environmental pollutants with genotoxic potential and for application in studies of environmental risk assessment and in hazardous waste evaluation.

The application of both genotoxic biomarkers DNA strand breaks and production of adducts DNA-PAHs used as genotoxicity assays are rapid and accurate techniques for determining the carcinogenic potential of environmental samples. Because the detection method is by fluorescent intensity it could easily be applied in the laboratory for different kind of environmental samples. The genotoxic potential of environmental samples for both biomarkers can be expressed as fluorescent relative intensity, upon normalization with control and reactive blank.

Both DNA strand breaks and DNA-PAH adducts analyses been extensively used to evaluate the toxicity of PAHs. Therefore, employing of DNA-SB and DNA-PAH adducts as biomarkers to assess the genotoxicity potential and bioavailability of PAHs and other organic compounds persistent from contaminated soil and water is feasible. The use of DNA fragmentation and adduct formation to estimate PAHs genotoxicity may provide additional insight into mechanism of action for complex organic mixtures.

The analysis of the DNA-SB and DNA-PAH adducts has been widely used to evaluate the genotoxicity of three PAHs. With the higher genotoxic effect for both test by B(a)P with respect ANT and PHE, situation that confirm many studies with others genotoxicity test like Ames test, micronucleus, sister chromatids and comet tests. However the sensitivity and speed of these test at present study are very important for process big number of samples.

So it is feasible, that they can be used as biomarkers, to evaluate the potential genotoxicity and indirectly the bioavailability of PAHs and other persistent organic compounds, air pollutants, soil and water. These results confirmed the mutagenic effect of B(a)P, related to a mechanism of oxidative damage to the DNA molecule, through both the induction of reactive oxygen species, as well as the metabolic activation of PAHs through the peroxidase enzymes, present in polymorphonuclear leukocytes.

The relationships obtained from both biomarkers justify the use of DNA fragmentation, as well as the formation of adducts, as adequate biomarkers of exposure to these genotoxic compounds. In addition, to support the use of DNA fragmentation and the formation of DNA-PAHs adducts to estimate the genotoxicity of PAHs and structurally related compounds, in such a way that they can provide complementary tools to elucidate the mechanism of action of similar compounds or genotoxic organic mixtures.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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