

## **Original Research Article**

### **Analysis of DNA damage biomarkers in human leukocytes by PAHs exposure.**

#### **ABSTRACT**

**Aims:** To study was evaluated 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:** Were using human leukocytes to the toxic cultures exposure to 24 hrs with different concentrations of anthracene, phenanthrene and benzo(a)pyrene. Were considered in the experimental design four toxic test groups for each one PAHs, control group, analytic blank group and standard fluorescence group.

**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.

**Methodology:** Isolation of the human leukocytes cells and Cell viability was verified by Trypan dye exclusion test, previously to the toxic cultures exposure to 24 hrs with different concentrations of anthracene, phenanthrene and benzo(a)pyrene. First was evaluated lethal concentration with neutral red (NR50) assay for each one PAHs was obtained. After were used sublethal concentrations range of these toxics for both biomarkers. In case of DNA fragmentation was used a fluorochrome to mark DNA fragments and isolation with alkaline solution finally determined with fluorescence spectroscopy. For other hand the formation of adducts DNA-PAHs was first isolated with a solvent system with polarity gradient and finally determined with fluorescence spectroscopy.

**Results:** PAHs with 3 aromatic rings showed lethaly cytotoxic and lower in case of the BaP with 5 aromatic rings. These results contrast with those observed between the DNA-PAHs adduction test, in which non-adduct formation was detected in Anthracene and Phenanthrene while in case of BaP was produced DNA adduction formation.

**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-PAH may be used as genotoxicity assays are rapid and accurate techniques for determining the carcinogenic potential of environmental samples.

**Keywords:** Genotoxicity biomarkers; DNA fragmentation; DNA adducts; PAHs; BaP; Anthracene; Phenanthrene.

## 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 is the air of the atmospheres of working of coal tar, formed by pyrolysis of coal in factories of gas and coke, which produce emissions of fumes from heated pitch. Generally, the content of benzo(a)pyrene (B(a)P) is maximum in the air at the top of the furnaces. The air at the top of the smoke and tar precipitator channels is extremely rich in this compound, having measured concentrations of up to  $500 \text{ mg/m}^3$  of B(a)P.

PAHs are formed by pyrolysis or incomplete combustion of organic matter that contains carbon and hydrogen. At high temperatures, the pyrolysis of organic compounds produced fragments of molecules and radicals combine to give rise to PAHs. The composition of the pyrosynthesis products depends on the fuel, temperature and time spent at high temperatures. Fuels that are PAHs 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, which can be turned to Quinones. PAHs react rapidly with oxides of nitrogen or  $\text{HNO}_3$ . For example, anthracene can oxidize to anthraquinone by action of the  $\text{HNO}_3$  or give a nitrogen derivative by a reaction of  $\text{NO}_2$  replacement [2].

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

The International Agency for research on Cancer (IARC), established in its 1983 report that had 11 polycyclic aromatic hydrocarbon carcinogenesis sufficient

evidence in experimental animals, also based on epidemiological studies showed a close relationship with the increase in the incidence of cancer in workers exposed to PAHs [4].

Anthracene also known as paranaftalene is a polycyclic aromatic hydrocarbon, according to the [5] U.S. Environmental Protection Agency, it is not classified as carcinogenic for human, 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 human (B2).

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

In the case of the PAHs main studied its genotoxicity mechanism is through the formation of adducts between DNA molecule and the metabolites of PAHs. Another no less important is the breaking of the molecule in a chain or double-stranded fragments, induced oxidative damage caused by the PAHs (Bolton et al., 2000), effect that is evident to be denatured DNA (Mendoza-Figueroa et al., 1985). The formation of fragments of the DNA chain can have a major impact on processes of mutagenesis and carcinogenesis. DNA adducts are considered sensitive biomarkers of both PAHs exposure and PAHs effect and are considered to be a cumulative index of current and past exposure to genotoxic compounds [7].

Carcinogenicity is believed to be due to members of a class of organic compounds known as PAH. 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 to forming DNA-PAHs adducts. It is generally accepted that chemical DNA adducts formation in organs susceptible to PAHs carcinogenesis is the first critical step in a multistep process that leads to cancer induction.

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

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 produced by the majority of the resulting metabolites of PAHs are epoxides with nearby diols attacking extranuclear amino groups of guanine and adenine [7]. In the metabolic pathway that follow the PAHs (taking as an example the B(a)P), from your income up to the formation of adducts is described by process beginning once PAHs entering the cell formed various epoxides by oxidative action of the mono oxygenase, microsomal cytochrome-dependent p450 (CYP1A).

These epoxies can regroup spontaneously in phenols, hydrolyze to hidrodials by the epoxido-hidrolases or join the glutathione covalently (spontaneously or catalyzed by glutathione-S-transferase). Phenols, some are oxidized to Quinones and others produce secondary epoxides (di-hidrodiolepoxidos), which are more reactive forms with DNA [10, 11,12].

## **2. MATERIALS AND METHODS**

### **2.1 Chemicals, Anthracene, Phenanthrene and Benzo(a)pyrene solutions**

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). ANT (97% pure) and BaP (98% pure) were purchased from the Kanto Chemical Inc. (Tokyo, Japan). Acquisition of 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, the concentrations of test to determine the lethal cytotoxicity were 0.02, 0.04, 0.06, 0.08 µM of Anthracene, 0.02, 0.04, 0.06, 0.08 µM of Phenanthrene and 0.5, 1.0, 2.0, 3.0, 4.0 µM of 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. The leukocyte fraction was suspended in RPMI-1640 culture media. Viability assessment to leukocytes 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 and BaP**

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

### **2.4 Determination of DNA fragmentation.**

For the quantification of the fragments of DNA chain was used the technique of fluorescence analysis of DNA unwinding (FADU) [15]. This technique is based on alkaline denaturation time-dependently the DNA under conditions of denaturation moderate using NaOH 0.1 M, since in alkaline conditions severe DNA is unwound completely. In alkaline conditions moderate is a process time-dependently, 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 adds the bisbenzamida 1.25  $\mu$ M and finally the determination by espectrofluorometry (Luminescence Spectrometer, model LS 50, Perkin-Elmer) [16]. The wavelength used were to emission of 450 nm and wavelength excitation of 355 nm and is carried out the results are expressed as relative fluorescence intensity (Fr).

### **2.5 Determination of adducts DNA-PAHs**

All samples were withdrawn with the culture medium and they are 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 espectrofluorometry (Luminescence Spectrometer, Perkin-Elmer, model LS 50) [15]. Adducts formation were estimated by fluorescence intensity at  $\lambda_{ex}$  = 417 nm using an excitation wavelength of  $\lambda_{ex}$  = 300 nm and the extent of adduction was calculated as the fluorescence relative intensity obtained previous correction with control and blank.

### **2.8 Statistical Analysis**

All tests were performed by triplicate. The values are means  $\pm$  standard deviation (SD). Single lineal regression analysis was used to determine the relation of concentrations of each PAHs with DNA strand breaks and adducts DNA-PAHs formation. All analyses were performed at  $p < 0.05$  was considered significant. Was using the software SPSS® v.22 [18] and the results are expressed as mean  $\pm$  standard error of the mean values of at least three experiments.

### 3. RESULTS

Lethal cytotoxicity to the ANT test yielded a  $LC_{50} = 0,352 \mu M$  ( $r = 0.94$ ,  $P < 0.01$ ), for the PHEN a  $LC_{50} = 1,521 \mu M$  ( $r = 0.94$ ,  $P < 0.01$ ), and an  $LC_{50} = 3.23 \mu M$  was obtained in the case of B(a)P ( $r = 0.94$ ;  $P < 0.001$ ). The lowest  $LC_{50}$  of the ANT and the PHEN, indicate that they have more potential cytotoxicity lethal, in the conditions of cultivation used neutrophil leukocytes.

Greater cytotoxicity of the ANT and PHEN with respect to B(a)P was observed, this can be explained based on the largest can lipophilic and low molecular weight both the ANT and PHEN, which allows these compounds to enter the cell and its organelles (mitochondria and nucleus) by transport passive [19].

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

There were no statistical differences in the levels of DNA-SB and DNA-ANT, expressed as fluorescence relative, under different exposure concentrations of anthracene, while B(a)P exposure induced a significant increase ( $p < 0.05$ ) in the production of DNA strand breaks as well as the adducts DNA production in all concentrations with respect to the negative control.

As can be seen in Table 1, there were no statistical differences in the levels of DNA fragmentation and formation of DNA-ANT adducts, expressed as relative fluorescence, with exposure to different concentrations of the ANT, while exposure of B(a)P induced a significant increase ( $p < 0.05$ ) in the production of DNA fragments, as well as in the formation of DNA-B(a)P adducts, as well as in all concentrations with respect to the negative control.

**Table 1. Results of biomarkers of DNA strand breaks and induction of adducts DNA-PAHs at different concentrations ( $\mu\text{M}$ ) of anthracene and BaP.**

Anthracene	DNA Strand breaks	DNA-ANT Adducts	B(a)P	DNA Strand breaks *	DNA-B(a)P Adducts*
$\mu\text{M}$	Rf	Rf	$\mu\text{M}$	Rf	Rf
<b>Blanco</b>	$0.091 \pm 0.05$	$0.083 \pm 0.05$	<b>Blanco</b>	$0.075 \pm 0.04$	$0.087 \pm 0.15$
<b>Testigo</b>	$0.291 \pm 0.05$	$0.226 \pm 0.11$	<b>Testigo</b>	$0.311 \pm 0.06$	$0.378 \pm 0.65$
<b>0.01</b>	$0.311 \pm 0.01$	$0.288 \pm 0.25$	<b>0.5</b>	2.127	$0.865 \pm 0.52$
<b>0.02</b>	$0.329 \pm 0.04$	$0.437 \pm 0.1$	<b>1</b>	4.304	$1.313 \pm 0.61$
<b>0.04</b>	$0.289 \pm 0.05$	$0.848 \pm 0.31$	<b>2</b>	6.822	$2.544 \pm 0.33$
<b>0.06</b>	$0.306 \pm 0.07$	$1.204 \pm 0.23$	<b>3</b>	12.859	$3.613 \pm 0.42$
<b>0.08</b>	$0.379 \pm 0.09$	$2.044 \pm 0.51$	<b>4</b>	23.41	$6.133 \pm 0.73$

Table 2 shows the results of DNA fragmentation, as well as the formation of DNA-HAP adducts, by sublethal exposure to PHEN and BaP concentrations. The results of both types of biomarkers are expressed as relative fluorescence intensity (Rf). As can be seen, the effect of DNA fragmentation due to exposure to PHEN showed no statistical difference with respect to the control, in the case of both types of genotoxic biomarkers, with exposure concentrations to human leukocyte cultures.

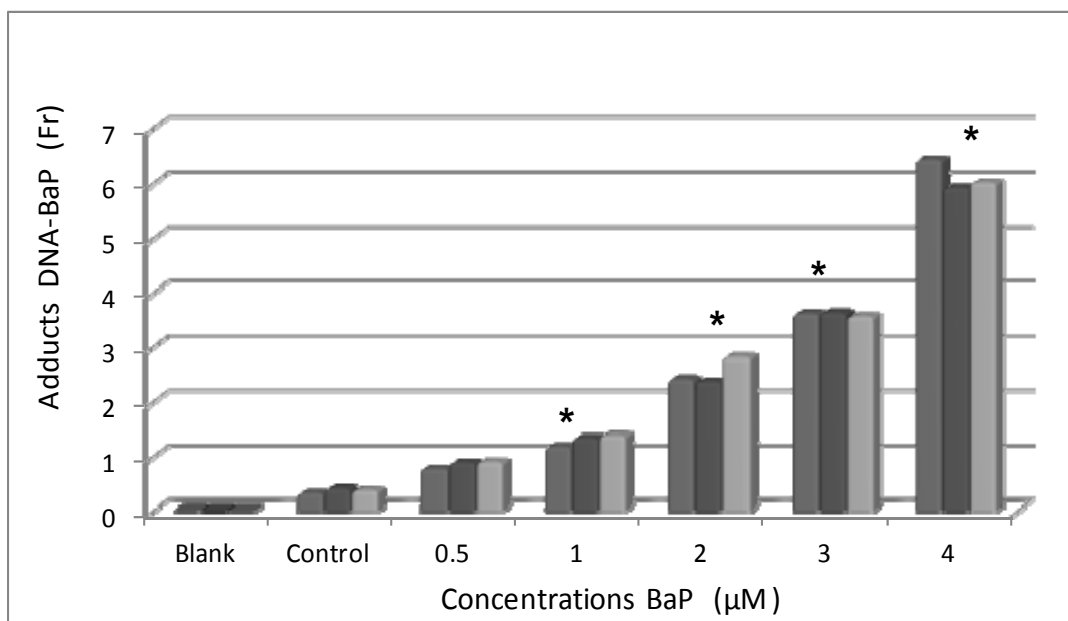
**Table 2. Results of biomarkers of DNA strandbreaks and induction of adducts DNA-PAHs at different concentrations ( $\mu\text{M}$ ) of phenantrene and B(a)P.**

Phenantrene	DNA strand breaks	DNA-PHE adducts	B(a)P	DNA strand breaks*	DNA-B(a)P adducts *
$\mu\text{M}$	Rf	Rf	$\mu\text{M}$	Rf	Rf
<b>Blank</b>	$0.087 \pm 0.03$	$0.087 \pm 0.05$	<b>Blank</b>	$0.075 \pm 0.04$	$0.087 \pm 0.15$
<b>Control</b>	$0.263 \pm 0.05$	$0.331 \pm 0.13$	<b>Control</b>	$0.311 \pm 0.06$	$0.378 \pm 0.65$
<b>0.01</b>	$0.327 \pm 0.08$	$0.388 \pm 0.25$	<b>0.5</b>	2.127	$0.865 \pm 0.52$
<b>0.02</b>	$0.298 \pm 0.04$	$0.417 \pm 0.1$	<b>1</b>	4.304	$1.313 \pm 0.61$
<b>0.04</b>	$0.336 \pm 0.04$	$0.448 \pm 0.13$	<b>2</b>	6.822	$2.544 \pm 0.33$
<b>0.06</b>	$0.367 \pm 0.07$	$0.381 \pm 0.17$	<b>3</b>	12.859	$3.613 \pm 0.42$
<b>0.08</b>	$0.327 \pm 0.09$	$0.421 \pm 0.16$	<b>4</b>	23.41	$6.133 \pm 0.73$

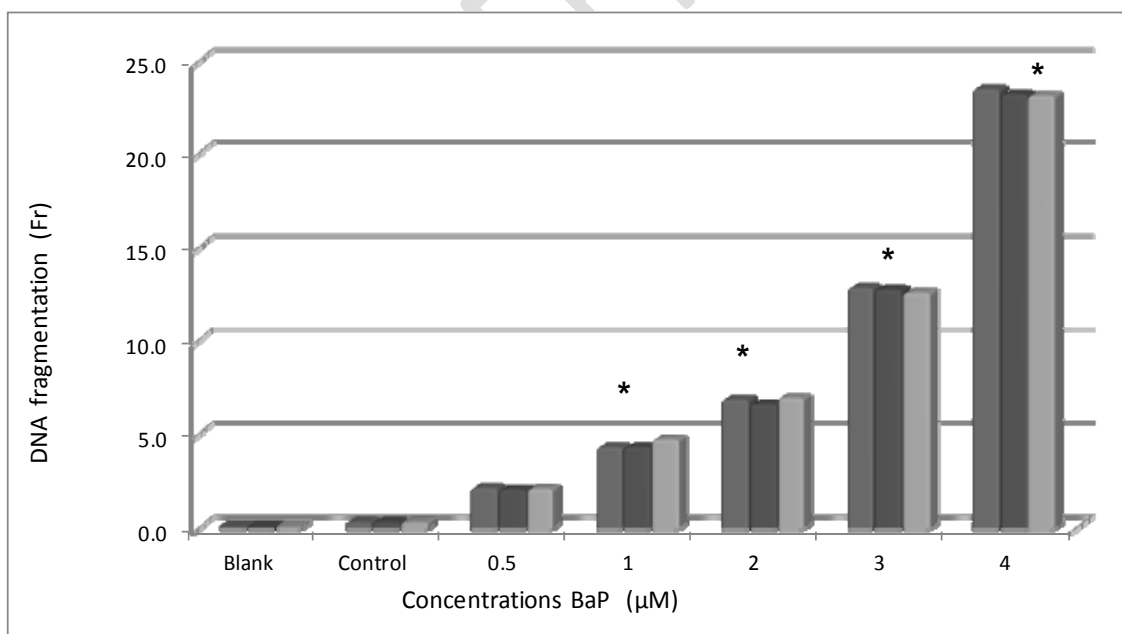
\*The groups marked with significant statistical represent the results triplicate

In the case of the formation induction of DNA-B(a)P adducts, statistical difference was observed ( $p < 0.05$ ) between the control and the highest concentrations of B(a)P (Figure 2), except for the lowest concentration. It is important to note that there is a proportional increasing relationship in the DNA response, with respect to the increase in B(a)P concentrations.





**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 by 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 compared to the negative control without toxic.**

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 (Figure 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.

In the Table 3 presents the results of the regression analysis of both the results of genotoxic biomarkers (DNA strand break and formation of DNA-PAHs adducts), after exposure to the anthracene, phenantrene and B(a)P. It was observed a higher coefficient of correlation between the formation of adducts with B (a) P ( $r = 0.96$ ), that with respect to the anthracene ( $r = 0.19$ ) and phenantrene ( $0.091$ ). A similar situation was observed with the 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 factors such as the molecular structure and the reflex on the properties such as the ionization potential. These results provide evidence that chloroperoxidase activity in the leukocytes mediated binding of PAHs to DNA occurs by one-electron oxidation fact showed by Marquez-Rocha et al. [17].

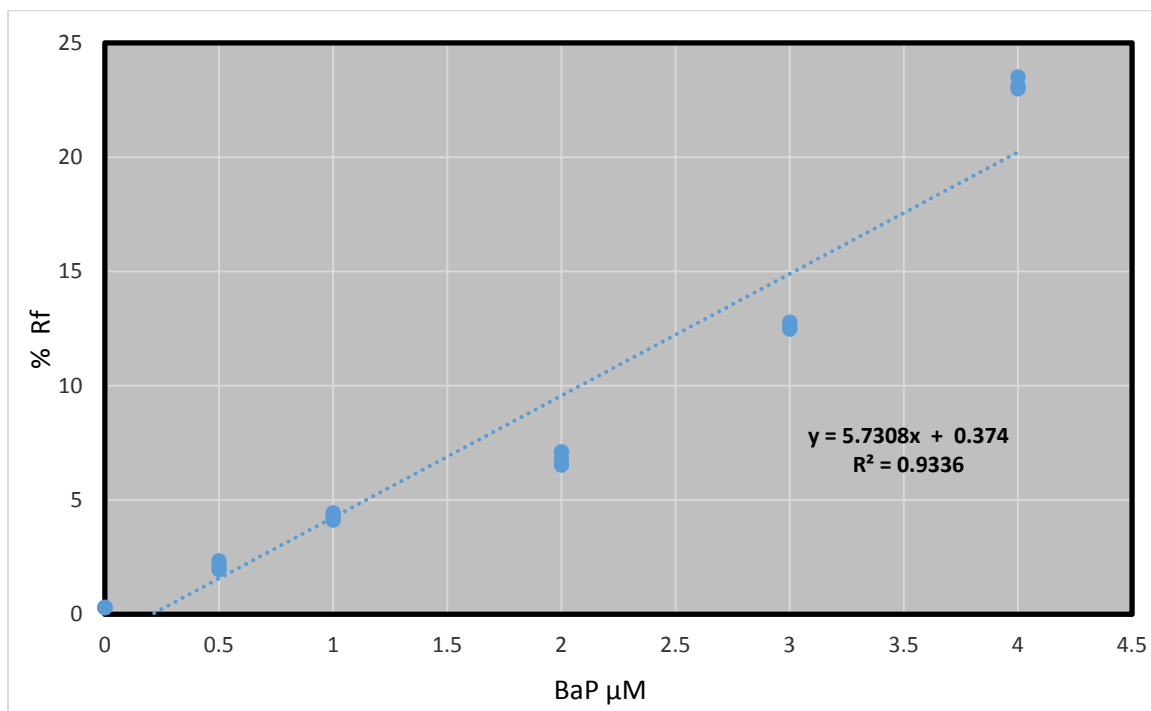
**Table 3. Parameters of linear regression between the PAHs and genotoxicity biomarkers**

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

\*The groups marked with significant statistical represent the results triplicate

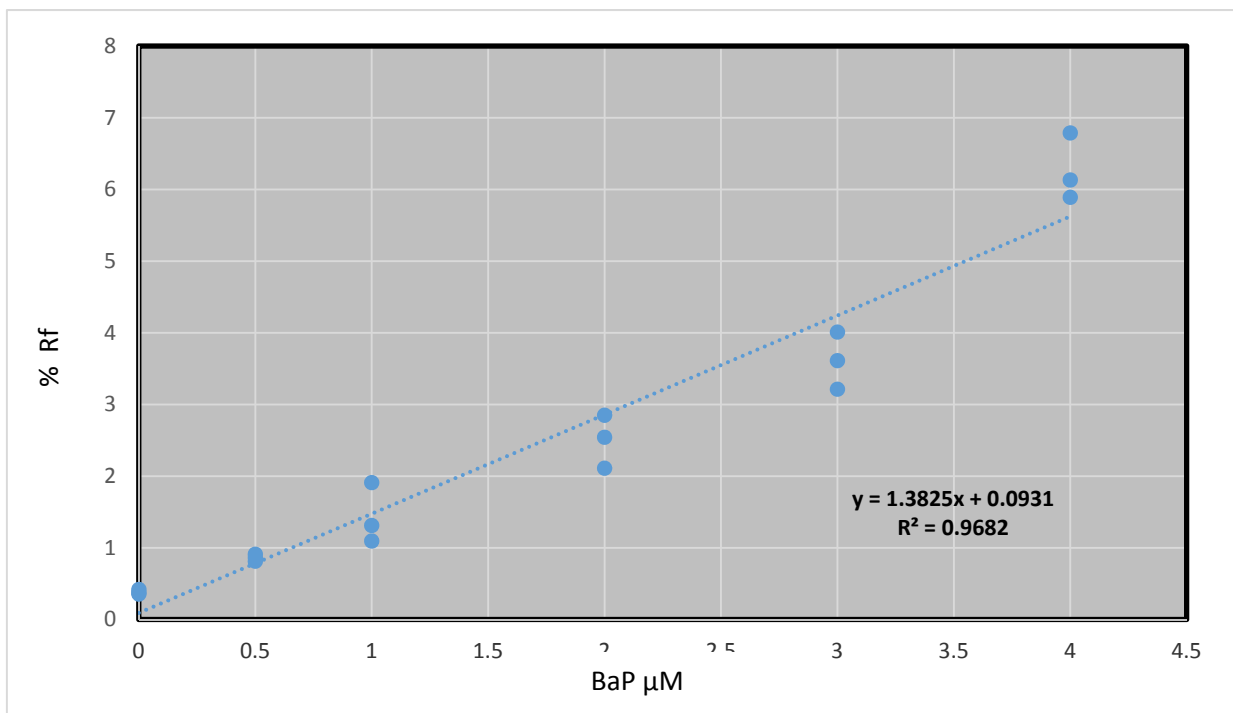
The linear relationship graphs corresponding to the exposure with B(a)P are shown below, since only for this PAHs compound, valid values of the linear model were obtained in terms of  $p < 0.05$  as well as  $r = 1$ .

In the figure 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 each  $\mu\text{M}$  unit of B(a)P, and a basal value of 0.37, in the absence of the toxic compound.



**Figure 3. Linear relationships of DNA strand breaks with B(a)P concentrations.**

In the Figure 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 each unit  $\mu\text{M}$  of B(a)P, and a basal value of 0.09, in the absence of toxic compounds.



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

#### 4. DISCUSSION

We found PAHs with 3 aromatic rings to be acutely cytotoxic and lower cytotoxicity was detected for PAHs with 5 aromatic rings. These results contrast with those observed with the DNA-PAHs adduction test, in which non-adduct formation was detected in Anthracene 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 and 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 mechanism of action for complex organic mixtures.

We found PAHs with 3 aromatic rings to be acutely cytotoxic and poor toxicity was detected for PAHs with 5 aromatic rings. These results contrast with those observed with the DNA-PAHs adduction test, in which non-adduct formation was detected in Anthracene and Phenanthrene with 3 aromatic rings; while B(a)P with 5 aromatic rings resulted in adduct formation.

The response of the biomarkers of genotoxicity, for the exhibition to Anthracene and Phenanthrene, 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 structure molecular and the grade of reactivity associated to the same, of such compounds, so the Anthracene and Phenanthrene not generated a response associated of them biomarkers, while in the B(a)P if is present, this 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 acute PAHs of 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 adduction 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. As 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 Cl 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 damage genotoxic.

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  $0.1\mu M$  of B(a)P. In considering that the level of basal fragmentation in the leukocytes analyzed it was 0.374% in the absence of B(a)P and that the EC50 corresponding to said ratio is  $1.07\mu M$  B(a)P (Table 3). Not so in the case of ANT and PHEN, a situation that can be explained in terms of the bioactivation [23]. These compounds to 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 radical and hydroxyl [24]. DNA fragmentation is produced by exposure to  $H_2O_2$  in the range of 25 to  $10\mu mol/L$ . Similar at the present study [25] observed that lymphocytes treated with concentrations of 0.6 to  $4.8\mu M$  of metabolite B(a)P dilepoxide 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  $30\mu g/ml$  of a B(a)P derivative for 24 hrs. Said 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/ $\mu$ g 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/  $\mu$ g 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 may be 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, previews normalization with control and reactive blank.

Both DNA strand breaks and DNA-PAH adducts and analysis has 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 PAHs. 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.



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