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Genotoxicity and Cytotoxicity Evaluation Applied to Environmental Health, Research of Polycyclic Aromatic Hydrocarbons

Ane Frohlich[1*], Dinara Jaqueline Moura[2] and Leiliane Coelho Andre[1]

[1]*Department of Clinical and Toxicological Analysis, Faculty of Pharmacy, Federal University of Minas Gerais, Av. Presidente Antonio Carlos, 6627, Zip code 31270-901, Belo Horizonte – MG, Brazil.*

[2]*Laboratory of Toxicological Genetics, Federal University of Health Sciences of Porto Alegre, Rua Sarmento Leite, 245, Zip code 90050-170, Porto Alegre - RS, Brazil.*

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***Corresponding author:** Ane Frohlich, Department of Clinical and Toxicological Analysis, Faculty of Pharmacy, Federal University of Minas Gerais, Av. Presidente Antonio Carlos, 6627, Zip code 31270-901, Belo Horizonte – MG, Brazil; E-mail: anebiomed@yahoo.com.br*

Abstract

Environmental toxicology contributes for the evaluation of chemical exposures, proposing interventions based in preventive actions promoting a risk decrease. The industrial development, agriculture activities, the increase of the number of automobiles, among others, lead to a constant increase in the concentrations of air pollutants that are hazardous to health, therefore becoming a public health problem. Besides many chronic and acute respiratory diseases, related to environmental chemical exposures, the consequences related to genotoxic effects are poorly evidenced. Biomarkers of genotoxicity such as the comet assay, micronucleus assay, Salmonella/microsome assay and chromosomal aberration assay are reliable techniques for the early detection of the damage and for the evaluation of the chemical exposure aiming the prevention, mainly in environmental exposures, in which there is a really short window between the exposure to the chemical and the appearance of the damage. This paper aims to identify potential biomarkers of genotoxicity as reference to environmental chemical exposure, its usage and contributions to the decrease of this public health problem, especially for the Polycyclic Aromatic Hydrocarbons (PAHs).

Keywords: biomarkers of genotoxicity; environmental health; PAHs;

Introduction: Health and environmental exposure

TEnvironmental health is established as the relation between the environment and the pattern of health o one population. This relation, according to the World Health Organization (WHO), involves all elements and factors that affect,potentially,health[1]. Therefore,new theoretical approaches among the relation production/environment/health are being studied aiming the discovery of the origin of this relation, pointing out new means of intervention based in preventive actions for the decrease of risk, having risk as the probability of the occurrence of an adverse effect, during an established exposition time [2,3]. Environmental toxicology aims to establish safety limits for a chemical agent, anticipating the consequences of damages caused to human health [4].

Concerning the environmental exposure to chemical factors, there are many potentially toxic agents dispersed in the atmosphere, those can be generated by anthropogenic sources, such as industrial and agricultural production, transportation means, generation of stationary energy and residential heating systems [5]. Most situations involve exposures to low concentrations, for a long period of time, resulting in an increase of health damages, which can appear only after a long time since the exposure. Therefore, making it very difficult to establish the causal link [6].

In this context, environmental pollution is a major problem concerning public health. It is classified as a mixture of many toxic substances and it is divided into four categories: volatile organic compounds, heavy metals, particulate material and in a ubiquous form composed by polycyclic aromatic hydrocarbons (PAHs). Most of these pollutants, such as the PAHs, present proven carcinogenic and mutagenic properties, since – once in the atmosphere – they participate in many chemical reactions, producing oxygenated and nitrogenated derivatives that are even more toxic than the original ones [7,8,9].

Although many studies revealed the diverse toxic effects of PAHs, that are still largely being studied; and the relation between cancer and the exposure to PAHs, the International Agency for Research on Cancer (IARC), has classified some of the PAHs as belonging to the group 1, due to human carcinogenic evidences, the regulation of exposure limits for PAHs in the environment is restricted to some countries, such as the United States, France, Italy, Germany and Switzerland [10]. Although the aforementioned countries include PAHs in their monitoring, some

differences are evidenced regarding the monitored members and their respective concentrations, although they agree on the unanimous monitoring of benzo(a)pyrene [11].

Biomarkers of genotoxicity, whose analyses allow the measure of the genetic and cellular damage, are becoming promising techniques for the toxicological analysis and for the evaluation of the cancer development associated with the exposure to chemical substances. Among all tests that can be performed for potential genotoxicity determination, are included the micronucleus assay, comet assay, chromosomal aberration assay and Salmonella/microsome [12,13,14]. Analyses of the genotoxicity biomarkers are parameters sensitive that allow a complementary approach for the environmental evaluation, when still in an early and reversible stage of damage. The approach and understanding of these biological analyses contribute as integral and motivational elements for an strategic and effective management, proposing the establishment of indicators of the quality of the air that are not evaluated by the competent authorities, becoming reference for the public politics of environmental health [15,2].

The genotoxic and carcinogenic effects of PAHs

The first publications on the carcinogenicity of organic combustion products were in 1775, when Percival Pott reported the high incidence of cancer in chimney sweepers in the city of London. These effects were later attributed to benzo(a)pyrene. However, with the advancement of the research, it was verified that the carcinogenic activity is related to the presence of a set of PAHs and nitro derivatives, and not to an isolated substance [16].

Therefore, air pollution, consisting of chemical substances adsorbed to the particulate matter has been the main environmental cause of death by cancer. The IARC announced, in 2013, that the air pollution is carcinogenic to humans, confirming its classification as group 1, based on scientific evidences. The particulate matter, consisted on PAHs in a complex mixture, was evaluated separately and was also classified as belonging to group 1 [17]. This scenario reflects a serious public health problem, with an estimated 6.4 million deaths from cancer caused by exposure to particulate matter contained in air pollution in 2015 [18].

Among many compounds formed by PAHs, sixteen are considered, by the Environmental Protection Agency-United States of America (EPA-USA), prioritary pollutants, due to its toxicological importance, which are: naphthalene, acenaphthylene, acenaphthene, fluorene, anthracene, phenanthrene, fluoranthene, pyrene, chrysene, benz(a) anthracene, benzo(b)fluoranthene, benzo(k)fluoranthene, B(a)P, indeno(1,2,3-cd)pyrene, benzo(g,h,i)-perylene, and dibenz(a,h) anthracene [10]. Mutagenicity and carcinogenicity related to PAHs exposure have been evaluated, taking benzo(a)pyrene as an indicator. Through this indicator, an evaluation regarding the exposure was established, allowing the calculation of the lifetime cancer risks (ELCR). It's from this indicator that the exposure assessment was established, making possible to calculate the excess lifetime cancer risks (ELCR). Therefore, the OMS disclosed the Risk Unit (RU) for PAHs mixtures, which was estimated to be 8.7x10-5 ng/m3 of benzo(a)pyrene. This means that there is an estimative of 8.7 cases of cancer related to benzo(a)pyrene exposure in a population of 100.000 people, when exposed to a concentration of 1 ng/m3. When the association between the B(a)P and the ELCR is needed for exposures above 1 ng/m3, the total level of B(a)Peq is multiplied by the RU [19, 20, 21].

During periods of cold weather, the exposure to PAHs is aggravated, due to the fact that there is an increase in the emissions, and those are allied to the influence in the atmospheric conditions. During the cold months, there is an increase of the mixed shallow layers and a frequent thermal inversion, whose phenomena is responsible for the decrease of the atmospheric dispersion and an increase of the sorption of the more volatile PAHs on the particles suspended in the air. On the other hand, during the warm weather, the atmosphere becomes more unstable, increasing the rainfall indexes, reducing suspended pollutants and favoring their photo degradation; throughout this period, the concentrations of PAHs are influenced by photochemical and oxidation reactions, which are triggered by the solar radiation, ozone and free radicals, decomposing them. Thus, meteorological conditions have contributed consistently in episodes that indulge the increase of risk of development of pathologies, especially lung cancer [22, 23, 24, 25].

In the particulate matter suspended the PAHs adsorbed are the ones with four or more aromatic rings, which are the major environmental concerns due to their mutagenic and carcinogenic properties. Nevertheless, in the atmosphere the PAHs react with other chemical substances, becoming more toxic then the original ones, due to the fact that, among atmospheric particles, it is estimated to exist more than 500 compounds of different chemical classes. However, the relation between the concentration of particulate matter and the mutagenic effects is not always direct, since many studies sustain the hypothesis that the mutagenic effect is related to the compounds present as a complex mixture, underestimating the amount of particles present in the environment. This idea is reinforced by the synergism of the reactions among the PAHs and other constituents of air pollution, in addition to the formation of nitro-PAHs, which have been proven to be very mutagenic and allow the formation of DNA adducts [26, 27, 28, 29, 30].

Due to PAHs low reactivity with biological systems, they are only activated via metabolization; generating reactive metabolites that can interact with macromolecules, inducing damage and producing Reactive Oxygen Species (ROS) and DNA adducts. One important reaction is related to the Benzo(a)Pquinone, a metabolite of B(a)P. Its presence is related to the increase of the cellular proliferation, making the signaling of the growth factor and increasing the ROS production [31, 32].

Cancer as a complex and multifactorial disease develops through genetic alteration. Oncogenes and tumor suppressor genes are emerging targets of genetic alterations, which are

involved in the cancerous process, comprising the epigenetic. Once the active metabolite o-quinones PAH is formed, it has the ability to interfere with the p53 tumor suppressor. In lung cancer this process is related to a mutation, which modifies the original function of p53, causing its inactivation, this relation is reported as dose-dependent [33].

Cell proliferation and migration are two important and implicit processes in metastasis and tumor progression. In a recent study, this relation was shown and it identified that the exposure to PAHs, mainly in the winter period, presented the capacity to stimulate the motility and invasion of tumor cells, these mechanisms involved ROS increase, degradation of the extracellular matrix and angiogenic signaling. Accelerated cell division, tumor formation, abnormal cell morphology, angiogenesis and metastasis are steps that comprise cancer and are reported as a consequence of this exposure [34,35].

Furthermore, several PAHs have demonstrated an association between carcinogenicity and immunosuppression. In addition to DNA damage, adverse effects include impaired serum immunoglobulin and cytokine levels, B-cell proliferation, surface antigen expression, increased apoptosis of peripheral blood mononuclear cells, increased natural killer (NK) cells, and altered phagocytic activity of monocytes. B(a)P as an indicator of PAHs demonstrates a participation in mechanisms that implicate an immune suppression, although it involves multiple processes that should be deeply evaluated [36].

Biomonitoring studies using biomarkers of genotoxicity contribute to the detection of damage caused by toxic substances, making it possible to identify the components attributable to genotoxic effects on human health by atmospheric pollution, allowing the understanding of the carcinogenic process. The proposal to associate biomarkers of genotoxicity with environmental exposure to PAHs and risk assessment in a predictive approach is a promising tool.

Characteristics evidenced in the presence of PAHs that are present in atmospheric pollution, such as the production of active toxic metabolites, DNA adducts, alterations in genetic material, production of reactive species, mutations, chromosomal aberrations among others studied effects can be effectively evaluated through Micronucleus Assay, Comet Assay, Ames Test and Chromosomal Aberration Assay. These means of evaluation are considered safe and informative, and complement each other analytical.

Biological effects and Susceptibility: Genotoxicity mechanisms

Genetic susceptibility contributes to the understanding of different metabolic responses, also influencing events of uptake, metabolism, excretion and binding of metabolites to the DNA or target proteins. The study on the exposure to PAHs present in air pollution, its metabolization and the patient's genetic susceptibility, considering the risk of cancer development, allows the identification of different sources of exposure, including new biomarkers [37].

Once PAHs are absorbed, distributed and accumulated in body tissues, mainly in lipophilic tissues, they are easily passing the cell membranes, by passive diffusion. The biotransformation of these compounds involves oxy-reduction reactions (catalyzed by mixed function oxygenases, cytochrome P450, NADPH-cytochrome c-reductase), hydrolysis (catalyzed by esterases), as well as conjugation reactions (catalysed by sulfotransferase, epoxide hydrolase, glutathione-S-transferase and UDP-glycosyltransferase), in order to increase the polarity of the oxygenated products, increasing its solubility in water, facilitating its excretion. On the other hand, the products of these metabolizing reactions can become more reactive, leading to the formation of products with toxic activity and causing genetic damages before being eliminated by the organism (Fig 1) [38].

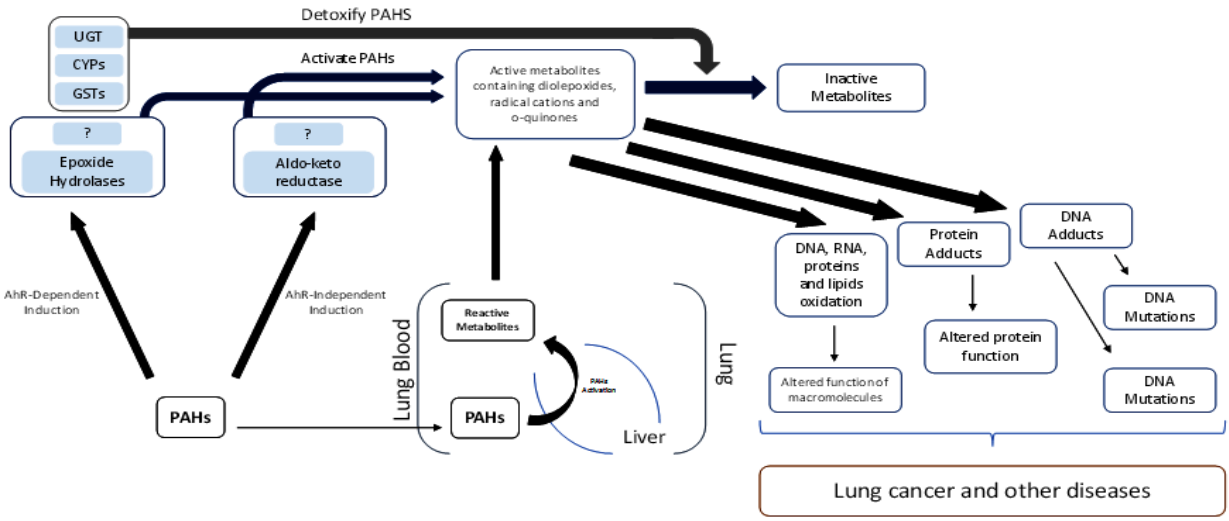

Figure 1: Mechanisms of DNA damage caused by the formation of toxic products from PAHs

The mechanisms that include the metabolization, by enzymatic activation of PAHs, involve three major pathways: CYP1A1/1B1 and epoxide hydrolase pathway (CYP/EH pathway), CYP peroxidase pathway and aldo-keto reductase pathway (AKR pathway) (Fig. 2) [39, 40].

The diol-epoxide formation proposes that the PAHs can be activated by the formation of reactive epoxides, while intermediate products interact with cellular constituents. This pathway involves three enzyme-mediated reaction. Firstly,

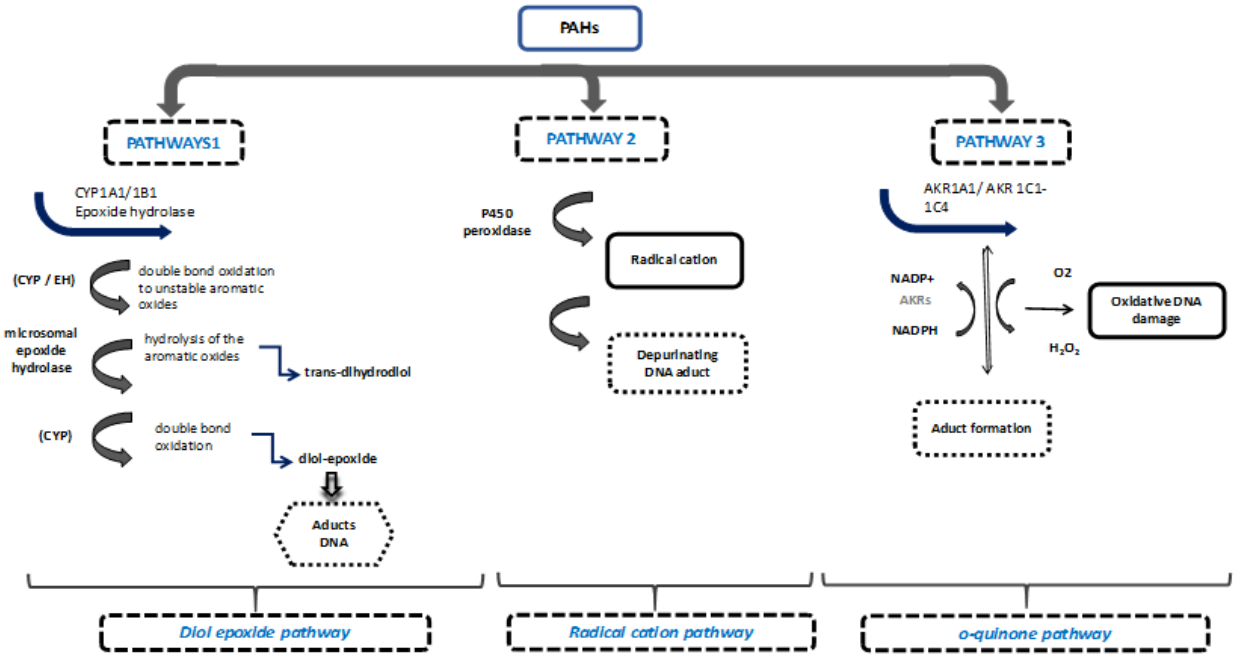

Figure 2: The known major pathways responsible for the metabolization and toxicity of PAHs

double bond oxidation catalyzed by cytochrome P450-dependent monooxydases (CYP / EH) occurs to unstable aromatic oxides. Thereafter, the hydrolysis of the aromatic oxides occurs by the microsomal epoxide hydrolase to trans-dihydrodiol. And finally, a second adjacent, CYP catalyzed, adjacent double bond oxidation with the function to generate the diol-epoxide. In the bay region the diol-epoxides are able to bind to DNA by their electrophilic character. The diol-epoxide reaction of some PAHs with the exocyclic amino group of 2'-deoxyadenosine (dA) and 2'-deoxyguanosine (dG), are structurally characterized and related to the process of tumorogenesis and formation of adducts [41, 42]. Most PAHs bonds are prochiral, being formed during the metabolic activation of stereoisomers, forming optically active products via CYP/EH metabolism, which can vary the PAH-diol-epoxide reactivity with the DNA, depending also on the number of aromatic rings involved, attributed to a greater ionization tendency [42].

Additionally, the CYP pathway is very well-established, allowing the activation of the diol-epoxide by the electrophilic route. In this pathway it is suggested that the cytochrome P450 dependant monooxydases, hydrogen peroxide (H2O2) dependant peroxides or H-prostaglandins synthases have the capacity to generate cation radicals formed through the oxidation of one electron. Also, there is a strong relation between the PAHs activity and the potential of ionization involved. The cation radicals formed by the metabolization generate a DNA damage and clearance at the apurinic sites, exceeding the cellular repair capacity, contributing to the mutation [41].

The third pathway involves the orto-quinones. Under physiological conditions, the dihydrodiol dehydrogenase (DD), which belongs to the AKR family, competes with the P450 in an oxidation process; this mechanism is NADPH+-dependant, involving the initial formation ketol that rearranges to form a catechol by the action of DD followed by autoxidation of the unstable catechol to o-quinone. The oxidation of NADPH+-dependent catalyzed by the DD is followed by the consumption of molecular oxygen and the production of hydrogen peroxide, occurring the formation of ROS in addition to o-quinone [40, 43].

In this moment, the agent is metabolized into a mutagen or to an intermediary product, usually electrophilic, reacting with macromolecules, such as proteins and target of DNA molecules. These biological alterations usually are reversible, however, in case they are not, there will be irreversible alterations, reaching the DNA content and manifesting itself as genetic mutations. Cells with alterations in the genetic information then initiate the growth process, leading the information of altered cell structures [44, 45, 46]. Then, PAH derivatives can be considered genotoxic and covalent adducts with DNA inducers. After interaction with

DNA, the cell triggers mechanisms of response to DNA damage, especially nucleotide excision repair (NER), responsible for the resolution of adducts toxic DNA agent. Two NER pathways are recognized: transcription coupled NER (TC-NER) restores the transcribed DNA strand in transcriptionally active genes, while global genom NER (GG-NER) ensures DNA repair in non-transcribed genome regions [47]. The difference between the NER pathways is seen at the lesion recognition step, while in TC-NER stalled RNA Polymerase II elicits the DNA damage response by recruiting several specific DNA repair factors including the Cockayne syndrome proteins A and B (CSA and CSB), in GG-NER the DNA lesions are recognized by two lesion recognition factors, XPC and DDB2 (XPE). After damage recognition both sub-pathways converge into a common damage verification step, catalyzed by proteins XPA, XPB, TFIIH and XPD by the generation of the pre-excision complex, then the XPG endonuclease incision at the 3' site and the ERCC1-XPF at site 5', after this DNA polymerase-PCNA complex catalyzes the addition of new nucleotides, and finally the action of the DNA ligase, completing the DNA repair process [48, 49]. However, it is observed that, in the presence of PAHs, the thermal stability of the DNA is compromised, with an increase in temperature, making it unstable. As the temperature increases, the repair capacity of the nucleotides decreases, and the process of recognition of the lesion fails [48].

As PAH damage is established, the signal is generated, increasing the activation and accumulation of p53, which plays an important role in cell regulation, because it is a transcription factor that regulates cell proliferation, differentiation, apoptosis and DNA repair [50]. In the cell, the p53 level is maintained by ubiquitin-mediated proteasomal degradation and Mdm-2 action. With the increase of p53 activation, activation of the caspases, release the cytochrome c, in the mitochondria, which promotes an alteration of the internal membrane of the mitochondria, generating permeability of the mitochondrial transition pores, causing a loss of cellular homeostasis, thus interrupting the synthesis of ATP and increasing the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). In addition, activation of 3-caspase causes DNA fragmentation and cleavage of specific cellular proteins such as actin, lamin, poly (ADP ribose) polymerase (PARP1) and fibrin, leading to apoptosis [51, 52].

ROS have an important role in the regulation of gene expression; if there is an imbalance between ROS and antioxidants, robust oxidative damage will occur, representing a serious impact on the organism, which is an indirect effect present in PAH exposure. Thus, base changes, mutations and/or DNA breaks occur. 8-Oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) is the most abundant base oxidation product of DNA, making it an excellent biomarker of effect in the evaluation of environmental exposure [53].

Although some works demonstrate that PAH induce ROS generation and DNA oxidative damage by different mechanism of action [54, 55], these damages occur and will be repaired by the base excision repair (BER). The BER pathway is initiated by one of at least 11 distinct DNA glycosylases, depending on the type of lesion, including OGG1 (8-oxoguanine DNA glycosylase) which repair 8-oxodG DNA damage, mentioned before. After initiation of BER by a DNA glycosylase, further processing may take place by "short-patch" BER, in which a single nucleotide gap is generated and subsequently filled and ligated, or by long-patch BER in which a gap of 2–10 nucleotides is generated and filled [56]. The major core proteins required in the different steps in short-patch repair after glycosylase include AP-endonuclease APE1, DNA polymerase β (Pol β), and DNA ligase I or III (LIG1/3). Already in long-patch repair uses replication proteins for processing subsequent to the glycosylase action and strand cleavage by APE1, which include DNA polymerase δ/ε, PCNA, FEN1, and LIG1 [57].

If ROS attack both DNA helixes, ruptures can lead to chromosomal changes, aberrations or chromosome translocations. Translocations are more serious because they are usually fixed in the genome and can lead to rearrangements of elements and genes, including oncogenes, thus increasing the risk in cancer development [58].

Importantly, both oxidative DNA damage and DNA adducts can initiate DNA double strand breaks (DSBs) when left unrepaired in cells replicating DNA. DSBs activate DNA-dependent kinases such as DNA PK, ATM, and ATR, which phosphorylate histone H2AX at the sites surrounding DSBs, leading to the recruitment of two major DSBs repair mechanisms: homologous recombination (HR), which is considered faithful; and less faithful non-homologous end joining (NHEJ) [59, 60].

The analyses of stable or unstable chromosomal aberrations have been used to evaluate groups exposed to the toxic substances present in the environment, mainly in large urban centers and industrialized regions, which have presented a higher incidence of these damages. The use of this analysis is evidenced in lymphocytes, which is validated and internationally recognized as an important biomarker of genotoxicity [61, 62, 63].

In addition to chromosome damage in human somatic cells, generated by long-term exposure to air pollution, genetic polymorphisms that may contribute to the low DNA repair capacity and to the variants associated with detoxification capacity reduction, in which mutations are responsible for the increasing susceptibility to such damages [64].

Individuals that have mutations in the glutathione-s-transferase M1 (GSTM1) and T1 (GSTT1) enzymes, when null have no enzymatic activity, these null genotypes correspond to a frequency of 50% in the Caucasian population. However, some polymorphic monooxygenases enzymes of cytochrome P450 have high enzymatic activity, the major cytochrome P450 variants of gene 1A (CYP1A1) include 2A, 2C, 2B and 4. Since the metabolism of PAHs depends on these enzymatic activities, these variant genotypes are associated with high rates of cytogenetic damage, evidenced by chromosomal aberrations in lymphocytes when exposed to air pollutants, increasing the risk of cancer [65, 66, 67, 68].

Considering that the environmental and individual response can be modeled by genotypic modifications, the presence of CYP1A1 plays an important role in the metabolism of PAHs and aromatic amines, converting them into prokarycinogenic ones. On the other hand, individuals presenting in their genotype the GSTT1, have an indicative of maintenance of genomic integrity [69, 70].

Also, significant differences in responses to exposure are evidenced between genders. In fact, the most intense occurrence is in the female gender, which in some studies have shown a higher level of CYP1A1 gene expression, supporting the hypothesis that female are particularly susceptible to the carcinogenic effects of PAHs in the lung [71].

Current studies contemplate the investigation of polymorphisms, which are essential for the understanding of individual susceptibility, generating more serious reactions to exposure to PAHs and therefore included in the investigation of environmental and individual effects under the influence of toxic substances. In addition to the aforementioned mutations, other mutation-related genotypes are described in this context, there is a positive association between DNA adducts and carcinogenic processes in the presence of SNPs mutations in XPD, GSTM1, Leu(432)Val, NQO1 C(609)T alleles, variants of GSTM1, GSTT1, GSTP1, XRCC1 399 Gln/Gln nucleotide variants and TP53 gene mutation [72, 73, 74, 75].

Genotoxicity tests

The genotoxic analyses present important results in the predictive evaluation, when it refers to the carcinogenic potential related to environmental exposure, contributing to the early diagnosis and identification of risk factors. Regarding the investigation of irreversible damages, techniques such as the micronucleus assay, chromosomal aberrations assay and Salmonella/microsome assay are used, those tests allow the identification of gene mutations, as well as the comet assay in the analysis of damages that are still reversible, these are contemplated in the genetic lesions [76, 77, 78].

This range of analyses is proven to be effective when used in the investigation of the toxicological effects of PAHs, since they may present heterogeneous biological responses, sometimes reversible or irreversible. Both responses can be worked on and identified in this exhibition; when the identification of adducts of proteins occurs, they do not present restorative capacity, reflecting a late exposure; in contrast to DNA adducts, these have the capacity to be repaired by the genetic machinery, although they are influenced by individual susceptibility, because when this process is not possible, mutation will occur [79].

However, genotoxic assays can be applied in a targeted manner, generating analytical advantage, in order to promote less expensive, more agile and early diagnosis. This vision is also shown by Tox21: Transforming Environmental Health, integrated by government research and testing agencies, the US Department of Health and Human Services and the US Environmental Protection Agency (EPA), which propose advancement in genomics, toxicity and assay techniques to improve the ability to assess chemically induced impacts that generate genetic damage, advancing research using other biological media, promoting less reliance on animal testing [76, 80].

Biomarkers of genotoxicity applied to healthcare

Micronucleus test

Micronuclei (MNs) consist of a cytoplasmic portion of chromatin that is located close to the nucleus, formed by acentric chromosome fragments or whole chromosomes, that get lost during the cellular division, resultant from the delay during the anaphase, therefore they are not included in the nucleus of the daughter cells, remaining in the cytoplasm of the interphase cells [81, 82, 83]. The occurrence of MNs is dependent on cell division and it cannot be used efficiently in cell populations that are not in division or in those that have a kinetics not well-known or well-controlled [84].

When the Best technical choice involves micronucleus, it is possible to evaluate the late exposure, evidencing chronic damage to DNA [55]. However, the use of this test implies an important limitation, the interindividual variability in the frequency of spontaneous MNs, due to an estimated occurrence of 2 to 36 MNs per 1000 cells. This fact is related to intrinsic factors, such as: diet, age and gender of the studied population, in addition to exposure to mutagens [85].

The MNs as biomarkers of genotoxicity are minimally invasive, which facilitates their manipulation; therefore, different biological matrixes can be used for in vitro analysis for this purpose, such as oral mucosa, different cell cultures, leukocytes, among others. In recent years a prediction has been made by the use of cell cultures to evaluate the genotoxicity of various substances; however, it is worth to emphasize the importance of making a careful selection of the experimental matrix, whose choice may compromise analytical fidelity and may result in false positives [86]

Considering that, the micronucleus test requires that the cells used are in cell division, the oral epithelium becomes an important tool for analysis, once it has a continuous renewal. Firstly, MNs are expressed in the basal cell layer, where mitosis occurs, after 7 to 10 days these cells migrate from the basal cell layer to the keratinized layer, becoming differentiated cells containing superficial epithelium and later are exfoliated [87]. Studies about the genotoxic effects related to atmospheric pollutants use this evaluation tool, turning the epithelial cells model systems of inhalation potential. In particulate matter extracts of 2.5 μm PAHs are found, which establish significant genotoxic effects, able to be seen through the numbers of MN [88].

The culture of HepG2 cells, derived from human cells, is also a promising option in the evaluation, since they have similar morphology to the epithelium cells, besides maintaining the

ability to synthesize and secrete most of the plasma proteins, characteristic of human liver cells. This cell line retains the activities of phase I enzymes such as cytochrome P450 CYP1A1, CYP1A2, CYP2B and CYP2E1, as well as phase II enzymes, including glutathione-S-transferases, sulfotransferases, glucuronosyltransferases and N-acetyltransferases, with a role in the activation and detoxification of chemical substances that react with DNA, which is necessary for the metabolization and activation of PAHs' toxicity [89, 90].

Recently, studies have demonstrated the functionality of MN, when testing the exposure of PAHs compared to the mixture of PAHs to metals, which are often coexisting environmental contaminants. Notably, the genotoxic effects were superior in the mixture of substances, suggesting synergism of reactions, confirming an overlap of effects on human and environmental health. The description of the evidence was reported by the increase in the number of micronuclei present in the evaluation of HepG2 cells exposed to the mixture, demonstrating the importance and especially the complexity of the environmental evaluation in mixtures of pollutants [90].

Comet Assay

The Comet Assay, also known as Single Cell Gel Electrophoresis (SCGE), is an invaluable tool for the investigation of damages to DNA. Originally created in 1980, to measure DNA breaks, soon underwent modifications for the detection of a large amount of lesions, being largely used due to its sensitivity, its large applicability in many different tissues and cells and also due to its small amount of sample required [91, 92, 93, 94].

This test consists of removal of the plasma content of the cells, leaving only the intact DNA, which remains at anchor points in the nuclear matrix, as a spherical structure containing DNA loops (nucleotide body). If there is DNA damage in the anchor regions, these loops become relaxed, visualized by the staining and electrophoresis field [95].

This assay analyzes the lysis of cell membranes, separating the super-annealed structure of the DNA, breaking the double helix, being allowed to migrate to the electrophoretic agarose matrix. When viewed under a microscope, the migrated cell takes the apparent shape of a comet, forming a "tail" containing fragments or DNA helixes. The comet analysis is based on the stage of fragmentation of the DNA and its migration throughout the microelectrophoresis, taking this as a crucial parameter for the analyses. The alkaline version, pH 13.0, described by Singh et al. (1988) is considered the most sensitive method, when compared to the neutral version, since it can detect more DNA damages, such as the break of a simple helix, damages to the alkali-labile sites, incomplete repair places and cross-links, therefore is the most used one [96, 97].

Comets are classified into five classes of damage. Class 0 corresponds to comets considered undamaged, class 1 corresponds to minimal damage, class 2 represents mean damage, class 3 severe damage and class 4 high damage. For damage classification three visual analysis indexes are used: percentage of damage classes, damage index and damage frequency, according to the formulas below [98].

Eq. 1: Damage Class Percentage = (n. of given class x 100) / total number of comets

Eq. 2: Index of Damage = (n. class 2) + 2x (n. class 2) + 3x (n. class 3) + 4x (n. class 4)

Eq. 3: Frequency of damage = [(n. total – n. class 0) x100] / n. total

Other parameters that are considered are head area, mean comet intensity, mean head intensity, mean tail intensity, tail length, tail height, percentage of migrated DNA and Olive's tail moment [99].

Eq. 4: Moment of free tail = product of the percentage of DNA in the tail x tail length

Modified Kite Assay may also be used. Simple changes of DNA bases can be detected by digesting the DNA nucleoid with specific enzymatic lesion that removes the base, leaving the apurinic / apyrimidinic (AP) site, that is converted into a stop associated with the AP lysis activity. The frequency of damaged bases is given by the increase of DNA breaks with the presence of specific endonuclease. In the present study, endonuclease III (EndoIII; thymine glycosylase glycosylase, EC 4.2.99.18), the DNA repair enzyme of Escherichia coli, was the first enzyme to be used [100]. Its application demonstrated for the first time an effect of antioxidant supplementation on endogenous DNA damage [101]. Formamidopyrimidine Glycosylase from DNA, or FPG (EC 3.2.2.23), in turn also used, removes oxidized purines, in particular 8-oxo-7,8-dihydroguanine (8-oxoGua) and formamidopyrimidines, i.e., adenine of or guanine - but also attacks guanine N7 adducts produced by alkylating agents [102,103].

Although the use of specific injury enzymes has great value for the comet assay, some care should be taken regarding the low stability of the enzymes with the use of critical concentrations as well as perform the use of cells that have known amount of damage as standard [95].

In some situations the DNA is concentrated almost entirely in the comet tail, referred to as hedgehog, where there is a possibility that definitive damage will be caused, leading to programmed cell death. However, it cannot be claimed to be cell apoptosis, for two reasons: apoptosis is irreversible and it is characterized by the effective fragmentation of DNA, certainly disappearing during electrophoretic lysis, which in fact does not occur in the comet assay [104].

Biomonitoring of PAHs exposure in large populations is an important advantage of the comet assay and represents the potential of this biomarker to facilitate advances in studies of large-scale mechanisms rather than observational studies. Although the comet assay is capable of assessing DNA damage,

additional studies complement the identification of the molecular mechanisms underlying DNA damage detected. Thus, due to the high concordance of the results with the comet assay in studies of human exposure to environmental pollution, parallel cytotoxic assays have revealed the importance of the complementary methodology [105].

Different interindividual responses are evidenced, in this case, it should be considered that some differences in sensitivity between MN and the comet assay may be recurrent to the type and repair capacity of the cells used, the cell cycle phase, the nature of the genotoxic studied and the duration between exposure and analysis. In addition, one must consider individual susceptibility, which is represented by different responses. In this way, the ideal is the complementation of these two techniques, allowing to define a wide range of genotoxic damages and a more careful evaluation, improving performance and analytical efficiency [104].

In studies of human biomonitoring for atmospheric pollutants using the comet assay, the population generally experiences multiple exposure conditions, which should be taken into account. Ideally, these conditions under study should be standardized in order to minimize the number of independent variables. As a priority in a validation study, it should be equipped with a wide knowledge about exposures to DNA-damaging agents and their interaction, an essential approach in the comet study involving environmental xenobiotics. In the analyses, the presence of intra-laboratory variability is unavoidable; this requires the inclusion of reference standards in each experiment, especially when the analysis comprises a long series of human cell samples over weeks or months [92].

Chromosomal Aberrations

Chromosomal aberrations are alterations that do not modify the number of chromosomes, but they determine structural abnormalities in these, which are classified according to the Protocol of Identification and Nomenclature of Aberrations (PAINT). In this protocol it is defined that, translocation is a chromosome in rearrangement with a centromere, presenting at least two colors; dicentric chromosome contains two concentric centromeres of different colors; acentric fragment is the linear part of red or green coloration without centromere; and insertion is an acentric chromosomal material inside the chromosome of another color. This definition is an important tool for standardization and cytotoxic evaluation [106].

PAHs are identified by their ability to modify DNA through o-quinones, the product of the metabolization process. Through PAH o-quinone, an indirectly related mechanism, the production of oxidative species (ROS) changes, which results in alterations of base, mutations or DNA breaks, seriously impacting on the exposed organism. These ruptures in the DNA molecule can generate chromosomal aberrations, evidenced by translocations, occurring gene rearrangement and formation of oncogenes [53]

In lymphocyte cell culture, the cytogenetic study and its alterations have contributed as biomarkers of genotoxic effect in PAH evaluations. The identification of the occurrence of high levels of chromosomal aberrations establishes an important predictive parameter, being associated to the increased risk of cancer in this environmental analysis [107].

Two variables, genomic frequency of translocations and percentage of aberrant cells are used to monitor PAH exposure, influenced by environmental pollution, which are covered by chromosomal aberrations. To do so, the use of Fluorescence in situ Hybridization (FISH) method demonstrates its functionality, since, as the concentration of PAHs increases during exposure, this method allows to identify the increase of the number of the two variables, besides determining the association between the frequency of translocations and the formation of adducts of DNA, corroborating to the sensitivity of the analyses that involve the cytogenetic study [108].

In a recent study using HepG2 cells, the metabolism of o-quinones generated by PAHs, via CYP1A1, was evaluated. It became clear that PAH-o-quinones have the ability to induce CYP1A1 expression, activated by expression of the gene response of the xenobiotic element (XREs), causing translocation of the aryl hydrocarbon receptor (AhR) in the nucleus. When hepatoma cells deficient in AhR translocation were tested, CYP1A1-inducing metabolism failed, confirming the dependence of AhR translocation on PAH depletion metabolism [43].

However, the association of the presence of chromosomal aberrations with factors linked to genotypic presence, which leads to different individual responses, attributed to susceptibility should be considered. A strong correlation is described between the high levels of cytotoxic effects with the reduced presence of the NAT2 and GSTM1 negative genotype, which is explained by the metabolic pathway of PAHs, which is mediated by enzymes members of the cytochrome P450 family and by glutathione-S-transferase and N-acetyltransferase. Consequently, with the decline of the genotypes corresponding to these enzymes, deficiency occurs in the detoxification process of the metabolic substances dependent on them, such as PAHs [109].

Salmonella/microsome assay

Salmonella/microsome assay was developed by Dr. Bruce N. Ames and collaborators in the 1970s and revised by Maron & Ames (1983). Different strains of Salmonella typhimurium, sensitive to substances capable of inducing mutation, are used for the test. This line presents the characteristic of auxotrophy to the amino acid histidine, as a function of mutations in genes of the route of biosynthesis of this amino acid, which makes them incapable of growing in culture medium with absence of histidine. In the presence of mutagenic substances, these cells revert to their character of auxotrophy, and become prototrophic, to synthesize histidine and, therefore, grow in media lacking this amino acid.

In addition to the mutation in histidine, Salmonella strains have genetic characteristics that give the analysis greater sensitivity in the detection of mutagenic substances, such as increased permeability of the bacterial wall by partial loss of the lipopolysaccharide barrier (rfa mutation) and deletion of the uvrB gene, decreasing the ability to repair [110].

Among the biomarkers of genotoxicity and mutagenicity, the Ames test has found great applicability in routine screening for chemicals and environmental samples due to its reproducibility, low cost and application in several samples in a short period of time. This assay demonstrates sensitivity, in an integrated approach, in studies to define DNA damage at environmental exposures, providing identification of the mutagen-related profile [111].

When analyzing samples of atmospheric material, the bacterial mutation tests have been valuable techniques and undoubtedly the most used ones, including environmental studies in large scales, considering multiple places and periods. It is observed that the fractions that contain PAH present an increase in mutagenic responses when the S9 fraction is present. However, PAHs contribute significantly to individual or population mutagenicity when exposed to air pollution, there is an association with the classes of polar compounds that contain nitroaromatic, aromatic amines and aromatic ketones, occurring the synergism of reactions [112].

Conclusions

Environmental health focuses on understanding that exposure is a health hazard; also it is the scope of assessing the magnitude of this exposure and interventions that can be implemented to reduce risk and prevent harm to human health. Environmental toxicology is bound to establish safety limits for chemical exposure to the environment and to evaluate and integrate the results of biomonitoring to support evidence-based data and decision-making.

New diseases appear every year and numerous cases are reported from environmental exposure, affecting human health and the environment. In contrast, scientific research and improvement of analytical technologies with efficient methods consolidated a complete evaluation protocol and integrated it to reality, in addition to providing understanding of the relation between exposure and biological effects.

Environmental regulation requires the incorporation of new biomarkers, consistent with the risk assessment and exposure of chemical contaminants. Thus, the development of trials, which can predict the early effects of genotoxicity and cytotoxicity, add advantages and complement the risk assessment, contributing to the definition of the environmental quality standard for health prevention actions.

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