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In vitro combined toxicity of hexavalent chromium, nickel and benzo(a)pyrene in human lung cells

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Abstract

Risk assessment of chemicals mainly relies on exposure to single chemicals and their hazardous effects, although the real scenario in occupational settings is more commonly characterized by exposure to chemical mixtures. Chromium (Cr), Nickel (Ni), and Polycyclic Aromatic Hydrocarbons (PAHs) are often present in occupational settings, such as aeronautic industries, where workers are exposed primarily through inhalation. Considering that those substances are recognized lung carcinogens acting mainly by genotoxic mechanisms, it is likely that interactive effects occur, indicating that the risk from occupational exposure to these chemicals should be assessed as a mixture. Such issue is being addressed in the HBM4EU Initiative, where a real scenario of occupational exposure has been studied.

In order to provide support to the hazard assessment of the referred mixture, the present work aimed to evaluate the combined toxicity of Cr(VI), Ni, and benzo(a)pyrene (BaP), using a human lung cell line (A549 cells). Cytotoxicity was assessed after 24 and 48h of exposure to different concentrations of each compound and mixture of compounds. MTT assay was performed for the different mixtures and for each individual chemical and a dose-response curve was established, enabling the determination of the IC_{50}, when possible. Genotoxicity was assessed through the micronucleus assay, after exposure for 24h, for single and mixture of compounds. The combined genotoxicity and cytotoxicity of the Cr and Ni mixture as well as that of Cr, Ni and BaP were determined comparatively to the single chemicals' toxicity to ascertain whether additive effects or deviations from additivity towards synergism or antagonism was obtained.

The results showed a high level of cytotoxicity for single Cr(VI) and single nickel; on the other hand, single BaP exposure didn't show a significant decrease in A549 viability. Genotoxicity results for the three compounds, all showed an increase in micronuclei frequency for all tested concentrations, confirming their genotoxic potential. Binary mixtures with Cr(VI) and nickel all caused a significant decrease in cell viability for both 24 and 48h of exposure, and modelling results with the CA model showed a weak antagonism whereas with the IA model, a synergistic effect was found for lower concentrations, with an alteration to antagonism at higher concentrations. Ternary mixture with Cr(VI), nickel and BaP also caused a significant decrease in cell viability, and although no modelling was performed, an apparent antagonistic effect was observed. At a genotoxic level, both binary and ternary mixtures caused a significant increase in micronuclei frequency, and modelling results for the binary mixture showed, with the CA model, an additivity effect and with the IA model, a synergistic effect. Once again for the ternary mixture, although no modelling was performed, an apparent antagonistic effect was found.

In order to consolidate the information and results presented in this work, it is necessary to explore more concentrations and different combinations. In addition, other relevant endpoints should also be explored, for example, epigenetic effects, ROS generation and effects on DNA repair.

Keywords: Mixtures; Risk assessment; occupational health; hexavalent chromium, nickel and PAHs.

Resumo

A avaliação de risco de diversos químicos baseia-se maioritariamente na exposição aos mesmos individualmente e aos seus efeitos adversos, apesar do cenário real, em âmbito ocupacional, ser caracterizado mais frequentemente pela exposição a misturas de substâncias. Dos efeitos que podem resultar dos diferentes tipos de exposição a misturas de substâncias, existem dois de extrema importância, o cancro e alterações genéticas que podem aparecer, não só na população exposta, mas também nos possíveis descendentes dessa mesma população. Tanto o Crómio, como o Níquel e os Hidrocarbonetos Aromáticos Policíclicos estão frequentemente presentes em âmbito ocupacional, como por exemplo na indústria aeronáutica, onde os trabalhadores estão expostos a estes químicos, principalmente através da inalação. Uma vez que essas substâncias são reconhecidas como carcinogénicas pulmonares, atuando através de mecanismos genotóxicos, é provável que ocorra um efeito interativo, indicando que o risco de exposição ocupacional a estes químicos devia de ser avaliado como uma mistura. No entanto, esta avaliação e interpretação é extremamente complexa e requer a intervenção de várias áreas distintas, tornando assim o estudo da exposição a misturas um trabalho demorado e com várias variáveis a ter em consideração. Esta problemática tem sido estudada na iniciativa do HBM4EU, onde um cenário verídico de exposição ocupacional tem sido estudado. Este é um projeto europeu de biomonitorização humana, que tem como principal objetivo avaliar a exposição humana, principalmente a nível ocupacional, a diversos químicos e os principais efeitos na saúde humana. Com este esforço, o projeto pretende criar uma ligação com as entidades legislativas, de modo a garantir que os dados obtidos possam ser utilizados para a criação de leis e medidas que minimizem a exposição aos compostos de risco. Dado que a exposição ocupacional se torna contínua e possivelmente com valores mais elevados de concentrações das várias substâncias do que a exposição ambiental da população em geral, é de extrema importância identificar os vários poluentes ocupacionais de interesse e intervir de modo a reduzir a sua emissão e promover a proteção pessoal da população exposta diariamente a substâncias de risco, no âmbito da sua profissão.

De forma a promover informação sobre a avaliação de risco da mistura referida, este trabalho tem como objetivo avaliar a toxicidade individual e conjunta do Crómio (VI), Níquel e Benzo(a)pireno, utilizando uma linha celular pulmonar humana (células A549), criando um modelo *in vitro* para a exposição ocupacional. Esta linha celular foi selecionada, uma vez que a exposição ocupacional a estas três substâncias ocorre principalmente por via inalatória. Numa fase inicial de trabalho, antes de serem realizados ensaios de genotoxicidade, é importante selecionar primeiro um intervalo de concentrações de cada substância, baseado na sua toxicidade. Os ensaios de citotoxicidade são usados precisamente para este fim e determinam os efeitos na viabilidade e proliferação celular. O ensaio de citotoxicidade utilizado foi o ensaio do MTT [brometo de 3-(4,5-dimetiliazol-2-il)-2,5-difeniltetrazólio] que foi realizado para cada químico individualmente, com um período de exposição de 24 e 48h, e foi determinada uma curva dose-efeito, permitindo a determinação do IC_{50}. A citotoxicidade (medida durante um período de exposição de 24 e 48h) e genotoxicidade (num período de exposição de 48h), medida através do ensaio do micronúcleo, da mistura binária de Crómio e de Níquel, assim como da ternária com Crómio, Níquel e Benzo(a)pireno foram também determinadas comparativamente à citotoxicidade e genotoxicidade individual de cada químico para perceber se foram obtidos efeitos aditivos ou desvios da aditividade para o sinergismo ou antagonismo.

Os resultados obtidos demonstraram níveis elevados de citotoxicidade para o Cr(VI) e o níquel, avaliados separadamente, com valores de IC_{50} de 3,30 μM e 1,28 μM para o Cr(VI), para os tempos de exposição de 24 e 48h, respetivamente. Para o níquel foram obtidos valores de IC_{50} de 0,51 mM e 0,22 mM, para os tempos de exposição de 24 e 48h, respetivamente. Para ambos os metais observou-se uma relação dose efeito. Por outro lado, a exposição ao BaP não provocou um decréscimo significativo da

viabilidade celular das células A549, ao contrário do que se esperava através da revisão da literatura, não demonstrando ser, a partir destes dados, uma substância citotóxica. Os resultados da genotoxicidade para as três substâncias individuais, mostraram um aumento significativo da frequência de micronúcleos para todas as concentrações testadas, confirmando o seu potencial genotóxico promovido, principalmente, pela formação de espécies reativas de oxigénio (ROS) em processos de destoxificação, causando níveis elevados de stress oxidativo nas células. Para além disso, os ROS têm também a capacidade de interagir diretamente com o DNA e provocar quebras e lesões no mesmo, detetadas através dos ensaios de genotoxicidade.

Todas misturas binárias, realizadas, de Cr(VI) e níquel causaram decréscimos significativos da viabilidade celular após 24 e 48h de exposição. Para além da análise direta dos dados obtidos para as misturas, foi também realizada a modelação matemática dos mesmos dados para a obtenção de informação relativa ao modo de interação das substâncias. Para isso foram aplicados dois modelos distintos, o "Concentration addition" (CA) e o "Independent Action" (IA). Ambos os modelos baseiam-se no modo de ação de cada substância. Se considerarmos que as substâncias têm modos de ação semelhantes, então o modelo a aplicar seria o CA, por outro lado se tiverem modos de ação diferentes, o modelo mais adequado é o IA. Dado que os processos pelos quais tanto o Cr(VI) como o níquel e o BaP causam toxicidade são muito complexos, diversificados e ainda alvo de grande estudo, seria reducionista limitarmo-nos apenas a um modelo matemático para explicar a interação das misturas. Sendo assim, optámos por utilizar os dois modelos mais reconhecidos atualmente e comparar a informação obtida através de cada um. No caso da mistura binária de Cr(VI) e níquel, os resultados da modelação matemática, para 24 e 48h, com o modelo CA indicaram um efeito fraco de antagonismo, por outro lado, com o modelo IA foi obtido um efeito sinergístico em concentrações baixas, alterando para um efeito antagonista nas concentrações acima do IC_{50} da mistura. No caso da mistura ternária de Cr(VI), níquel e BaP, houve também um decréscimo significativo da viabilidade celular após 24 e 48h de exposição. Neste caso, como apenas foi testada uma mistura, não houve dados suficientes para a realização de uma modelação matemática adequada, no entanto, a partir dos resultados obtidos podemos inferir empiricamente que a toxicidade das três substâncias foi inferior ao esperado da soma das suas toxicidades, mostrando então um efeito antagonista da mistura.

Ao nível genotóxico, as misturas binárias causaram aumentos significativos na frequência de micronúcleos e os resultados da modelação através do modelo CA mostraram um efeito aditivo, e com o modelo IA, foi obtido um efeito sinergístico. Com a mistura ternária, novamente existiram aumentos significativos na frequência de micronúcleos e devido à falta de dados para uma modelação matemática adequada, já mencionado anteriormente, apenas foi possível fazer uma avaliação empírica dos efeitos da mistura. Considerando que cada químico, por si, tem a capacidade de induzir micronúcleos, se os seus efeitos fossem aditivos seria de esperar uma frequência de micronúcleos muito elevada na mistura, o que não foi o caso. Sendo assim, e tendo por base esta observação podemos inferir que estamos perante um desvio para o efeito antagonista da mistura de Cr(VI), níquel e BaP.

De modo a consolidar a informação obtida neste trabalho, um maior número de concentrações e, por consequência, um maior número de combinações deverá ser explorado futuramente, em próximos trabalhos relativos ao tema, de modo a aumentar o poder da modelação matemática e poder corroborar ou retificar os resultados obtidos nesta dissertação.

Palavras-chave: Misturas; avaliação de risco; saúde ocupacional; crómio hexavalente, níquel e HAPs.

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List of abbreviations and acronyms

AHH-1 – Human B lymphocyte cells

AhR – Aryl hydrocarbon receptor

AKR – Aldo-Keto Reductase

ARNT – Aryl Hydrocarbon Receptor Nuclear Translocator

ATCC – American Type Culture Collection

A549 – Adenocarcinomic human epithelial lung cells

BaP – Benzo(a)Pyrene

BEAS-2B – Human lung epithelial cells

CA – Concentration Addition

Ca^{2+} - Calcium ion

Caco-2 – Human colorectal adenocarcinoma cells

CBMN - Cytokinesis-blocked micronucleus assay

CBPI - Cytokinesis-blocked proliferation index

CO_2 – Carbon dioxide

Cr(III) – Chromium trivalent

Cr(IV) – Chromium dioxide

Cr(VI) – Chromium hexavalent

CYP – Cytochrome P450

CYP1A1 – Cytochrome P450, family 1, subfamily A, polypeptide 1

Cyt-B – Cytochalasin b

DBS – Double strand breaks

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

EDTA – Ethylenediamine tetraacetic acid

EGFR – Epidermal growth Factor Receptor

EPA – Environmental Protection Agency

FBSi – Fetal Bovine Serum inactivated

Fpg – Fluorescent plus giemsa

GSH – Glutathione

HBM4EU – Human Biomonitoring for Europe

HepG2 – Liver carcinoma cells

HIF – Hypoxia inducible factor

HK-2 – Human kidney cells

HSP90 – Heat shock protein 90

HT-29 – Human colorectal adenocarcinoma cells

H1975 – Human lung adenocarcinoma cells

H2AX – H2A histone family member X

H3K9Me2 – Histone H3K9

IA – Independent Action

IARC – International Agency for Research on Cancer

LS-174T – Colon adenocarcinoma cells

MCL-5 – Human lymphoblastoid cells

MG-63 – Human osteosarcoma cells

MLH1 – MutL homolog 1

MLO-Y4 – Murine osteocyte-like cells

MMC – Mitomycin C

MN - Micronucleus

MoA – Mode of Action

MTS – [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]

MTT – [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium]

NAD(P)H – Nicotinamide adenine dinucleotide phosphate

NBUDs - Nuclear buds

NER – Nucleotide excision repair

Ni - Nickel

Ni_3S_2 – Nickel subsulfide

$NiCl_2$ – Nickel chloride

$NiCO_3$ – Nickel carbonate

NiO – Nickel oxide

NiS – Nickel sulfide

$NiSO_4$ – Nickel sulfate

NPBs - Nucleoplasmic bridges

NR – Neutral Red

OECD – Organisation for Economic Co-operation and Development

PAHs – Polycyclic Aromatic Hydrocarbons

PBMC – Peripheral blood mononuclear cells

PBS – Phosphate Buffered Saline

p32 – Mitochondrial protein

RBL-2H3 – Rat basophilic leukemia cells

RI – Replication index

ROS – Reactive Oxygen Species

Rpm – Rotations per minute

RPMI – Roswell Park Memorial Institute medium

SAEC – Human small airway epithelial cells

SDS – Sodium dodecyl sulfate

TK6 – Lymphoblastoid cell line

V79 – Chinese hamster lung cells

WHO – World Health Organization

XAP2 – Hepatitis B vírus X-associated protein

Introductory note

This thesis contains data and/or methodologies presented in the following communications or scientific meetings:

Poster Presentation

Alves, I., Ladeira, C., Silva, M. J. (2019). *Occupational exposure to mixtures and the related health effects – The case of Chromium (VI), Nickel and Polycyclic Aromatic Hydrocarbons.* Jornadas ToxOmics, 28th October 2019.

Alves, I., Ladeira, C., Tavares, A. M., Viegas, S., Louro, H., Silva, M. J. (2020). *In vitro combined toxicity of chromium, nickel and PAHs in human lung cells as support for mixtures assessment*. 3rd Workshop on Human BioMonitoring in Portugal, 18th November 2020.

Tavares, A. M., Alves, I., Moreira, R., Louro, H., Ladeira, C., Viegas, S., Loureiro, S., Santonen, T., Göen, T., Kortenkamp, A., Luijten, M., Silva, M. J. (2021). *Occupational exposure to metals and PAHs: combining literature-based exposure and in vitro hazard data towards a mixture risk assessment*. EUROTOX virtual congress, 27 September-1 October 2021.

1 Introduction

1.1 Environmental and occupational exposure to chemicals and health effects

One of the greatest scientific concerns nowadays focuses on exposure to various chemicals considered toxic and harmful to human health. Within the effects that can result from different types of exposure, there are two that stand out for their extreme importance, cancer and genetic changes/defects that appear in the exposed population and also in the possible descendants of that same population, respectively (Parker, 2014). About 90-95% of cancers result from exposure to endogenous and exogenous agents. The later are associated with, personal behaviors and habits, such as smoking, diet, infections, exposure to solar radiation, stress, obesity, low physical activity and include also, environmental pollutants present in the air, water, soil, among others (Anand *et al*., 2008). These pollutants are released into the environment, not only from natural activities, but mainly from anthropogenic activities, the impact of these last ones are of high relevance due to the occupational exposure of a considerable portion of the affected population to industrial pollutants. According to the World Health Organization (WHO), about 19% of all cancers are directly attributed to environmental factors described above, and in particular to environmental pollutants or biological agents included in the occupational exposure (Prüss-Üstün *et al*., 2006). Only 5 to 10% of cancers are attributed to genetic factors and the remaining are caused by interaction of environmental and genetic factors (Anand *et al*., 2008).

Given that the occupational exposure becomes continuous and possibly to higher concentrations of chemicals than the environmental exposure of the general population, it is extremely important to identify the occupational pollutants to be able to reduce their emission, and protect the population daily exposed in the workplace.

1.2. The HBM4EU project

This dissertation was developed under the scope of the European project, "Human Biomonitoring for Europe" (HBM4EU). This project aims to understand and evaluate the human exposure to various chemicals, including occupational exposure, using human biomonitoring and the consequent effects on health. It is a project in force since 2017, involving the European Environmental Agency and 30 participating European countries, being Portugal one of them (Bopp *et al*., 2018). HBM4EU in addition to assessing exposure to chemicals in different European countries and their effects on human health, seeking to improve risk assessment, also aims to establish a platform for Human Biomonitoring in Europe. The main purpose of this platform is to harmonize the various methods and activities of biomonitoring among the countries involved, so that the data obtained can be comparable at the EU level and identifying exposure pathways and upstream sources (Bopp *et al*., 2018; Santonen *et al*., 2019). The substances or groups of substances were selected based on public concern, scientific evidence and the urgency to regulate in order to protect occupational and consumer health (Sarigiannis *et al*., 2019).

With this effort, the project hopes to be able to create a connection with the legislative entities, in order to ensure that the data obtained can be used in the creation of new laws and measures that reduce the exposure to the various hazardous compounds identified, including in occupational settings (Bopp *et al*., 2018; Ganzleben *et al*., 2017). In view of these concerns, an occupational study focused on exposure to hexavalent chromium [Cr(VI)] in several industrial sectors and activities was carried out. Furthermore, as workers are not exposed to single substances but to a mixture of substances in some sectors/activities, exposure to a mixture of Cr(VI), nickel (Ni) and polycyclic aromatic hydrocarbons

(PAHs) was identified as better reflecting occupational exposure in some industrial sectors, e.g., the aeronautic sector.

1.3. Human exposure to chromium and associated effects

Chromium can exist in 11 states, from Cr(IV) to Cr(VI). Trivalent chromium [Cr(III)] and hexavalent chromium [Cr(VI)] have a major environmental significance because of their stability (Saha *et al*., 2011). Chromium compounds are also occupational contaminants, primarily generated from industrial processes. Environmental chromium enters the air through burning of fossil fuels, waste incineration and discharges of chromium waste in water (Nickens *et al*., 2010). Even though it is present in the natural environment, it is in an occupational context that chromium deserves more attention and where it acquires greater relevance (Santonen *et al*., 2021). Workers and the population in general are mostly exposed by dermal and respiratory routes, being the last one the primary route of exposure. Tobacco smoke, including electronic cigarette smoke (Williams *et al*., 2017), is the most important source of exposure of the general population. However additional exposure routes include oral ingestion of contaminated water and plants that bioaccumulate this metal, i.e., through food chain (Nickens *et al*., 2010; Saha *et al*., 2011).

Chromium is widely used in the compound manufacturing industries, in metal alloys, in the treatment and leather coloring, welding, among others (SCOEL, 2017). It is estimated that the main uses for this metal are metallurgical (67%), refractories (18%), and chemical industries (15%) (Saha *et al*., 2011). It is estimated that occupational exposure to chromium compounds may affect 30000 workers annually, and chromium exposure related diseases are a major concern among those industrial workers (Annangi *et al*., 2016).

Cr(VI) hazard is well documented and the published data supports its genotoxicity and mutagenicity *in vivo* and *in vitro* and its consequent carcinogenicity (Nickens *et al*., 2010). Cr(III) is generally considered less genotoxic than Cr(VI) mostly due to poor membrane permeability to it and because it is rapidly excreted into urine when compared to Cr(VI) (Zhang *et al*., 2011). In its trivalent form, chromium acts as an essential micronutrient important in some physiological functions which leads to a big discussion between several authors who claim that Cr (III) is not as benign as thought and can be in fact genotoxic. Fang *et al.* demonstrated that Cr (III) induces DNA degradation, like Cr(VI) in some type of cells, including the yeast and mammalian Jurkat cells. In fact, results from that specific study, showed that Cr(III) ability to generate DNA damage was significantly greater than that of Cr(VI) (Fang *et al*., 2014). This is a controversial topic that definitely needs further investigation.

When inhaled, Cr(VI) accumulates in the bronchioles, resulting in deposits. Here the detoxification processes result in dissolved Cr(VI) or small particles that have the capacity to penetrate the cell membrane through channels/transporters. After entry into the cytoplasm, Cr(VI) suffers a metabolic reduction to its intermediate states, with lower levels of oxidation, Cr(V) and Cr(IV), and to the final stable reduced trivalent form, Cr(III). This reduction process is mediated through the action of intracellular components such as glutathione (GSH), ascorbate, NAD(P)H, some proteins, among others. From these reductant agents, ascorbate and GSH have a greater impact in this process (Nickens *et al*., 2010). In addition, some biologic fluids, such as saliva, gastric juice and tissues, such as liver and lung parenchyma, are able to reduce Cr(VI) (Proctor *et al*., 2014). In this reduction process, different free radicals are generated, mainly reactive oxygen species (ROS) (Zhang *et al*., 2011). The excessive production of ROS may cause oxidative stress which can lead to gene expression dysregulation, different cellular redox state and DNA and proteins damage. It is hypothesized that the process of carcinogenesis can be triggered by the described events (Nickens *et al*., 2010; Saha *et al*., 2011). In summary, the toxic

effect of hexavalent chromium originates mainly from its oxidizing power, due to the formation of free radicals during its reduction to trivalent chromium and other intermediates (figure 1.1).

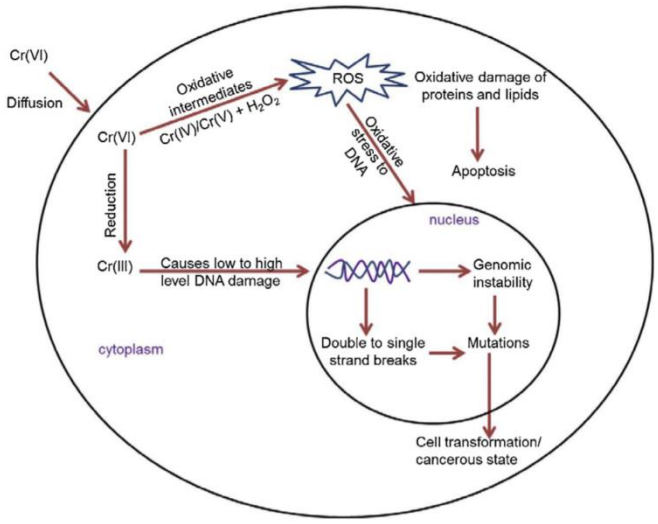


Figure 1.1 - Intracelular mechanisms explaining how chromium acts as a genotoxic compound. Annangi et al., 2016.

As described before, the level of genotoxicity of Cr(III) when compared to Cr(VI) is still very ambiguous, but many studies have proven that the trivalent form of this compound has in fact the capacity to bind to DNA. Due to its poor membrane permeability it cannot act by itself, but when it is originated already inside the cell, through the reduction process, Cr(III), Cr(IV) and Cr(V) can interact directly with the DNA and cause DNA adducts that lead to several genotoxic damages, such as DNA single- and double-strand breaks (Nickens *et al.*, 2010; Fang *et al.*, 2014).

The most common forms of DNA damage caused by Chromium compounds are:

- Breaks in one or both strands of DNA, mainly derived from oxidative damage;
- Binary or ternary Cr-DNA adducts are the most frequent ones. These adducts consist of a chemical element or a chemical group covalently linked to one or more DNA nucleotides. Among these, DNA interstrand or intrastrand cross-linking has been reported (figure 1.2). The latter affects the phosphodiester bonds between the two DNA strands and represents one of the most common genetic lesions in mammalian cells, caused by Cr(VI). Binary adducts are the most common form, representing about 50% to 75% of all Cr-DNA adducts (Saha *et al.*, 2011; Zhang *et al.*, 2011; Fang *et al.*, 2014), although DNA-Proteins cross-links have also been reported.

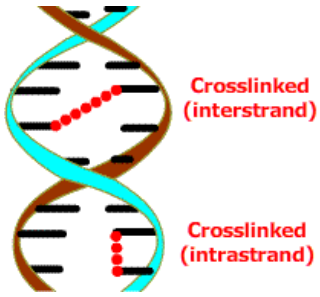


Figure 1.2 - Representation of DNA interstrand and intrastrand cross-linking. Adapted from: RnCeus, 2019.

The genotoxicity and possible carcinogenicity of different hexavalent chromium compounds differs significantly. In occupational environment, increase lung cancer risk is more pronounced in industries with exposure to less soluble Cr(VI) compounds. This is due to the longer period of permanence in the respiratory tract, namely, in the lung. The accumulation will also be higher, as well as the dose of this compound that will penetrate and affect the target tissue (Proctor *et al*., 2014).

Strontium chromate, calcium chromate and zinc chromate are some examples of Cr (VI) compounds that are relatively insoluble and that have the greater carcinogenic potential (Saha *et al*., 2011).

In addition to being one of the causes of cancer in the respiratory tract, inhalation of hexavalent chromium can also cause perforation of the nasal septum, asthma, bronchitis, pneumonitis and inflammation of the larynx. Outside of the respiratory tract, these compounds are also related to allergies, dermatitis, dermis necrosis and skin corrosion, as well as the development of gastric (Deng *et al*., 2019) and colorectal cancer (Saha *et al*., 2011; Sciannameo *et al*., 2018). Due to these characteristics, chromium is currently one of eight metals among the 50 priority toxic substances in the list of toxic substances by the Agency for Toxic Substances and Disease Registry. This metal, in addition to being mutagenic, is also considered by the International Agency for Research on Cancer (IARC) to be carcinogenic to humans when it is in its hexavalent form, currently making one of 33 compounds listed to pose the greatest potential health threat in urban areas (Nickens *et al*., 2010).

1.4. Human exposure to nickel and associated effects

Ni is a metallic compound and it is the twenty-fourth most abundant element in the earth's crust. This metal is widely used in the industry to make stainless steel and other metallic alloys (80%); electroplating (10%); foundry applications and printing inks (5%). Besides its use in industry, large amounts of Ni are released to the environment as a result of the industry processing, but also by natural phenomena such as volcanic eruptions (Cameron *et al*., 2011). Citizens, in general, might be exposed to this metal by ingestion of contaminated food or water, smoking, and dermal contact with nickel-containing products, like jewelry and coins. Even though there are many different routes of exposure to this substance, once again inhalation is the primary route of exposure and the most relevant in the occupational context (Cameron *et al*., 2011). Since the major route of exposure is the inhalation route, some aspects have to be considered when depositing and absorbing the nickel particles at the respiratory tract level. The chemical and physical form of this element, and especially the aerodynamic diameter of the particles, are the main characteristics that influence this parameter. Only about half of the nickel particles with an aerodynamic diameter less than 30 μm are inhaled by humans, and from this fraction, the larger particles (5 μm-30 μm) are deposited in the nasopharyngeal area. On the other hand, small particles (1 μm-5 μm) manage to reach the trachea and bronchioles. Only particles with an aerodynamic diameter less than 1 μm reach the alveolar region of the lungs (Schaumlöffel *et al*., 2012).

The bioavailability of nickel for various organisms, especially for humans, and the biochemical effects caused by it, depend a lot on the species and forms of the element. The different forms of Ni include elemental nickel (Ni), nickel oxide (NiO), nickel chloride ($NiCl_2$), nickel sulfate ($NiSO_4$), nickel carbonate ($NiCO_3$), nickel monosulfide (NiS), and nickel subsulfide (Ni_3S_2) (Cameron *et al*., 2011; Schaumlöffel *et al*., 2012). At the absorption level, of nickel-based compounds, the soluble compounds are those that are most quickly absorbed. The absorbed nickel is subsequently distributed throughout the body through the bloodstream. Although the soluble nickel compounds are the most easily absorbed, the least soluble compounds are known to be stronger carcinogens (Cameron *et al*., 2011). This is because soluble forms are easily dissolved in the mucosa and nickel ions are removed quickly. On the

other hand, less soluble forms enter the cells by phagocytosis and are slowly dissolved, promoting a continuous source of nickel ions (Schaumlöffel *et al*., 2012). It is believed that the carcinogenic potential of nickel is proportional to its ability to increase intracellular levels of nickel ions (Cameron *et al*., 2011). At the molecular level, nickel does not behave like a strong mutagen because it does not show high affinity for DNA, though it can form DNA cross-links and can indirectly (through ROS formation) induce DNA strand breaks, leading to chromosomal aberrations and most importantly disrupt DNA repair system by inhibiting enzymes required for DNA repair (Cameron *et al*., 2011; Zambelli *et al*., 2016). These observations have led to the hypothesis that the carcinogenicity associated with Ni compounds is mediated by their epigenetic effects, ultimately, deregulating gene expression. Indeed, many studies have shown that Ni binds with high affinity to nuclear proteins, for example, histones (Schaumlöffel *et al*., 2012). Nickel ions are able to induce heterochromatinization and iniciate chromatine condensation (Genchi *et al*., 2020).These complexes Ni-heterochromatin lead to alterations such as condensation and silencing of gene expression caught in silenced chromatin regions, by the induction of DNA methylation (Cameron *et al*., 2011; Schaumlöffel *et al*., 2012; Genchi *et al*., 2020). As result of gene silencing, cells are altered to a higher state of neoplastic transformation. In addition, the ability to bind to proteins and other components facilitates the production of ROS which, once again, leads to oxidative stress that has been linked with carcinogenicity (Cameron *et al*., 2011).

In addition, experimental studies have shown that nickel compounds are able to interact with Ca^{2+} sensing receptors. This interaction may induce hypoxia-inducible factors which leads in the first place to cell death, but may enhance cell potential to survive in anaerobic environment, enabling previously initiated cancer cells to progress and evolve to a full malignant state (Cameron *et al*., 2011).

Regardless of the nickel species in question, exposure by inhalation to high doses over relatively short periods of time, can induce several lung diseases and damage to the nasal cavity and mucosa. Pulmonary irritation and inflammation, emphysema, pulmonary cell hyperplasia, fibrosis and allergic asthma, bronchitis and pulmonary edema are some of lung diseases associated with nickel exposure (Schaumlöffel *et al*., 2012; Jose *et al*., 2018). Outside the respiratory tract, anemia, pancreatic, kidney and prostate cancer are also common as a result of exposure to high concentrations of nickel (Jose *et al*., 2018).

According to IARC, nickel compounds are considered carcinogenic to humans (IARC, group 1), while nickel as a metal or used in metal alloys is only considered a possible carcinogen (IARC group, 2B).

1.5. Human exposure to polycyclic aromatic hydrocarbons and associated effects

Polycyclic Aromatic Hydrocarbons (PAHs) are composed of two or more aromatic rings, with low solubility in water, being extremely lipophilic. These chemicals always occur in complex mixtures in the environment and the composition of such mixtures depends on the type of material and the conditions under which the same material was combusted. They are formed mainly by the incomplete combustion of organic materials during several activities, such as traffic, cooking, volcanic eruptions and carbonization. Tobacco smoke also contains high concentrations of PAHs. Because of such widespread sources, PAHs are present almost everywhere (Danuta *et al*., 2017, 14). The major sources of these substances are in occupational context, particularly, in industrial processes associated with burning of oil and coal. The highest levels of exposure are observed in aluminum production, followed by roofing and paving and the lower levels are observed in coal liquefaction, coal tar distillation, wood impregnation, chimney sweeping and in power plants (IARC, 2010a, 2012). Human exposure occurs mainly through inhalation and skin contact. About 500 PAHs have been detected in the air, but most

measurements have been related with one PAH in particular, Benzo(a)pyrene (BaP), which has been used for many years as a model in various bioassays and has also been used as a model substance for the carcinogenic PAHs present in the environment (WHO, 2010). Plasma levels of BaP were found to be higher in humans living in urban industrial areas than in outer suburban places, confirming its main release through industrial processes and traffic emissions (Larsen, 1995).

After entering the body, through inhalation, PAHs are transported on the ciliated mucosa and may penetrate into the bronchial epithelium cells (WHO, 2010). There, PAHs undergo one or two stages of metabolism. Phase I is characterized by the introduction of a polar reactive group making it a suitable substrate for phase II. In this second phase, endogenous substituents, such as sugars or amino acids, are added increasing water solubility so this metabolite can be easily excreted. This is described as a detoxication process, but along with this process, several reactive intermediates/metabolites are generated. These intermediates may be much more toxic than the parent compounds and may be able to establish covalent bonds with DNA, forming adducts (Danuta *et al*., 2017). These PAH-DNA adducts, if not properly removed by repair systems, lead to the induction of mutations that activate proto oncogenes or inactivate tumor suppressor genes, both of which are of great relevance in cancer development (WHO, 2010). In addition, during biotransformation ROS can also be generated, inducing oxidative stress which, as previously seen, is an important and relevant factor in the occurrence of DNA damage and mutations that may be related to carcinogenic processes (Danuta *et al*., 2017). In summary, some PAHs are considered mutagenic and carcinogenic because reactive intermediates are formed upon biotransformation, e.g., diol-epoxides that bind to cellular macromolecules, particularly, DNA leading to DNA lesions, gene mutations and chromosome alterations (WHO, 2010). The liver is the organ with the highest metabolizing capacity, followed by lung, intestinal mucosa, skin and kidneys (Anderson *et al*., 1989). Besides lung cancer, BaP and other PAHs exposure is associated with bladder, skin, pharynx, esophagus and larynx cancer (Danuta *et al*., 2017).

BaP belongs to group 1 carcinogens according to IARC, as well as all products containing this hydrocarbon in conjunction with others. Recent environmental studies have identified PAHs of considerably higher toxicity than the 16 priority PAHs classified by the Environmental Protection Agency (EPA), however there is still a lack of studies in most of these PAHs to justify their inclusion in routine measurements (Stec *et al*., 2018).

1.6. Mixtures

The scientific community has shown an increasing interest in evaluating and, if possible, quantifying multiple adverse health effects caused by numerous combinations of different substances with toxic properties (Tsatsakis *et al*., 2017). In fact, the population is exposed, throughout life, to an increasing quantity and variety of chemicals and compounds, simultaneously. This exposure isn't constant and has different doses of the various compounds, depending of the type of exposure (Hernández *et al*., 2017). This is one of the main issues that makes the whole problem around mixtures extremely difficult to evaluate and to predict its consequences.

Therefore, when assessing mixtures toxicity instead of their constituents individually, it is possible to obtain a more realistic approach of the potential of these substances to promote some diseases, since, in the real environment, these compounds present themselves mainly in mixtures with others (Taylor *et al*., 2016). This assessment of the combined effects of substances to which humans are co-exposed or cumulatively exposed throughout life (aggregated exposure) also provides the opportunity for competent (national or international) regulatory authorities to publicly intervene. In occupational settings the protective measures can rely, for example, on the implementation of individual

protection measures or can go further and contribute to the proposal of new occupational exposure limits to different substances. Moreover, new methodologies, in particular computational methodologies/tools, have been developed to assist in the assessment of mixtures and have generated new and meaningful data on the mechanisms underlying chemical mixtures effects (Hernández *et al*., 2017). This last point is extremely important because regulatory requirements for risk assessment of these mixtures exist for intentional mixtures such as commercialized formulated products, but there is no regulatory provision for non-commercial artificial mixtures that represent the scenario of real-life exposure, whether it is occupational or environmental (Hernández *et al*., 2017). The main obstacle to accomplish this goal is that achieving exposure scenarios simulating real life is a complex issue since the exposure to multiple chemicals may lead to a web of interactions and mechanisms that result in diverse health outcomes. This type of evaluation has to be seen as a multifactorial and multidisciplinary work, and it needs the collaboration of different scientific fields for an adequate treatment and integration of the data and results obtained (Hernández *et al*., 2017). The outcomes of exposure to any kind of compounds are not only limited to occupational or environmental exposure. There are other factors linked to the individual's lifestyle that can be determinant for the development of certain diseases or in the level of severity of the consequences of the exposure. In addition to environmental factors, individual habits, such as smoking, or even the individual genetic makeup, introduce variability and become another difficulty and another factor to be considered when interpreting and assessing human health outcomes from exposure to mixtures of toxic compounds (figure 1.3) (Liu *et al*., 2012).

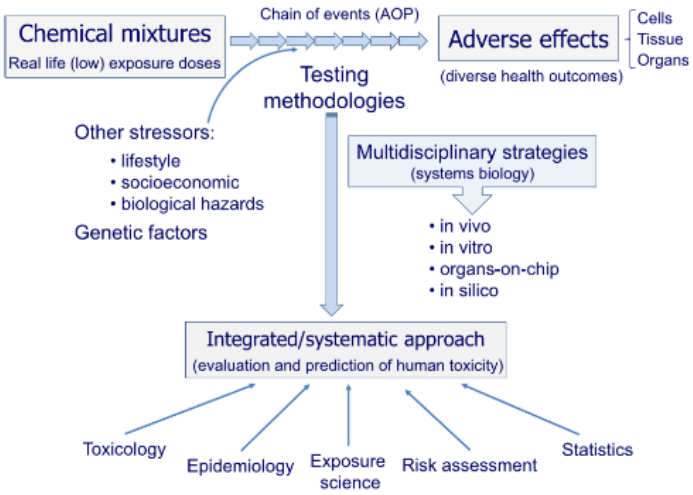


Figure 1.3 - Multiple hazard approach for long-term health outcomes and integrated approach of multiple lines of evidence for toxicity testing and prediction (AOP: adverse outcome pathways). Hernández *et al*., 2017.

At the results evaluation level there are some concepts that need to be understood to define toxicological interactions, such as additivity, synergism and antagonism.

For the assessment of chemicals mixtures toxicity, two reference mathematical models have been successfully applied, concentration addition (CA) and independent action (IA). CA has been generally used at the most conservative approach and according to this model, the joint action of a mixture is the summation of individual toxicities. It also assumes that the components of the mixture share the same mode of action (MoA). On the other hand, IA model assumes different MoAs and it's based on the hypothesis of a dissimilar action of mixture components, usually to assess if the probability of toxicity of one chemical is independent from the probability of toxicity of another (Pinhão *et al*.,

2020; Tavares *et al*., 2013; Jonker *et al*., 2009). In summary, this means that the relative effect of a toxic chemical remains unchanged in the presence of another chemical. Both models assume that chemicals don't interact and deviations from the predictions indicate interaction and induction of synergism/antagonism patterns. Synergism occurs when the combined effect is greater than the sum of the individual effects of each compound (additive effect). Antagonism is the opposite, it presents a weaker effect than expected from an additive effect (Ermler *et* al., 2014).

In an occupational context, more studies need to be performed in order to assess mixtures toxicity. The mixtures exposure scenario is well documented in ecotoxicology studies, where Cr(VI) and Ni were found to have greater than expected toxic effects in several different studies (Mo *et al*., 2016; Martin *et al*., 2021).

The mixtures of Cr and Ni are likely to occur in some industrial sectors, e.g., welding, while exposure to Cr, Ni and PAHs are less frequent. Nevertheless, they may occur, for example, during waste incineration and in the aeronautic industry. In the latter, some parts of the aircraft fuselage are coated with a metallic alloy containing Cr and Ni while the repair of the engines in the same open space generates the diesel exhaust rich in PAHs (Peng *et al*., 2015).

1.7. Assessment of cytotoxicity and genotoxicity of chemical substances and mixtures

1.7.1. Cytotoxicity assays

Before performing genotoxicity tests it is important to select a concentration-range of the test substance based on its toxicity. The cytotoxicity assays are usually used to determine effects on cell viability or proliferation, or direct effects that lead to cell death, caused by the tested substances.

MTT Assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide) assay is one of the most used colorimetric assays for cell viability assessment, and it was the first homogeneous viability test adapted to 96 wells (Mosman, 1983). It is based on the ability of living cells to reduce tetrazolium into purple insoluble crystals of formazan, through dehydrogenase and mitochondria enzymes and some reductor agents such as NAD(p)H (Stockert *et al*., 2012; Riss *et al*., 2013). Through this reaction, it is possible to infer the mitochondrial activity. Since for most viable cells, mitochondrial activity is constant, any change in the viable number of cells is linearly related to mitochondrial activity. This alteration is detected measuring formazan concentration through absorbance measurements (Van Meerloo *et al*., 2011). The signal generated by the MTT assay depends on several parameters, namely the MTT concentration, the incubation period, the number of viable cells, among others. Despite the practicality and relevance of this assay, some factors that can lead to misinterpretation of results must be considered. The reduction of MTT can be exacerbated by external factors such as some drugs, antioxidants or even inhibitors of cell proliferation that may fail to fulfill the expected objective and, on the other hand, stimulate and affect the reduction of MTT and compromise the true results of cytotoxicity and cell viability (Riss *et al*., 2013).

1.7.2. Genotoxicity assays

Cytokinesis-blocked micronucleus assay (CBMN)

The cytokinesis-blocked micronucleus assay (CBMN), commonly called as *in vitro* micronucleus (MN) assay is a genotoxicity assay used on a large scale to asses chromosomal alterations induced, in vitro, by numerous environmental agents. It is also frequently used as an effect biomarker in human biomonitoring studies. This widespread use is mainly due to the fact that it is suitable for more basic studies exploring the mechanisms of action of some agents, both physical and chemical, but also for screening studies of various substances such as pesticides and environmental contaminants. It is an easy test to apply and to follow the protocol, is a fast assay and with relatively low costs. It is also suitable for different cell types and its results show that it can be a good alternative to other more complex genotoxicity assays, such as the chromosome aberration assay. The MN assay is capable of distinguishing between two different types of effects, clastogenic effects (chromosome breakage) and aneugenic effects (abnormal number of chromosomes) (Sierra *et al*., 2014).

This assay can measure, not only the frequency of micronuclei, but also other nuclear anomalies such as nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs). All these anomalies are biomarkers of genotoxic events and chromosomal instability. For this reason, it is called also as the MN cytome assay (Fenech *et al*., 2011).

A micronucleus includes chromosome fragments or whole chromosomes surrounded by a membrane. It appears as a "third nucleus", in the cytoplasm of binucleated cells, when observed under a microscope. MN can be originated, during anaphase, from acentric chromosomes (chromosomes fragments without centromere) or chromatid fragments caused by misrepair or unrepaired DNA breaks, or can be originated by whole chromosomes that didn't bind to the mitotic spindle, and weren't taken to opposite poles during anaphase (figure 1.4). NPBs are originated during anaphase when the centromeres of dicentric chromosomes are pulled to opposite poles of the cell during mitosis. In the absence of breakage of the anaphase bridge, the nuclear membrane surrounds the daughter nuclei and the anaphase bridge and forms a NPB. The NBUDs are characterized by having the same morphology as a MN, with the exception that they are connected to the nucleus by a stalk of nucleoplasmic material. NBUDs represent the process of elimination of amplified DNA, DNA repair complexes and possibly excess chromosomes from aneuploid cells (Fenech *et al*., 2011).

Since MN are expressed in cells that completed a cell division, they should be scored when this cells are in the binucleated stage. In order to achieve that, cytochalasin-B (cyt-B) is used. It is a mycotoxin able to block cell cytokinesis, obtaining as binucleated cells the ones that suffered only one cell division in culture.

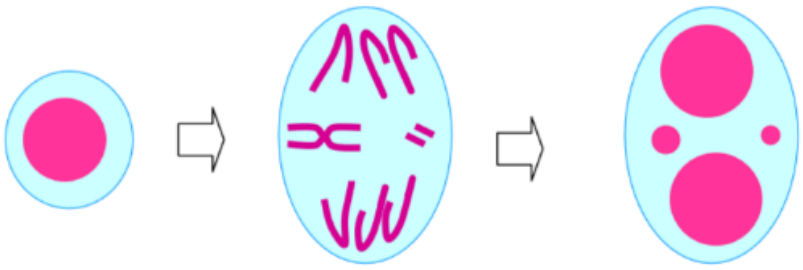


Figure 1.4 - Micronucleus formation. Adapted from Fenech *et al*., 2011.

2. Objectives

Currently the assessment of risks from exposure to toxic chemicals is mainly focused on exposure and toxic effects of single chemicals. However, exposure scenarios in occupational settings are generally characterized by an exposure to a complex mixture rather than to single chemicals. As such, the health effects from exposure in several workplaces and while performing different activities are probably associated to the mixture effect than to each contaminant effect per se. Chromium (Cr) and Nickel (Ni), as heavy metals, and polycyclic aromatic hydrocarbons (PAHs) are linked with an increased risk of a wide range of events, namely lung cancer. However, the effects from co-exposure to Cr, Ni and PAHs need to be addressed as a mixture in an occupational context.

The aim of this project, which is part of the HBM4EU Project, is to develop an *in vitro* study that mimics an occupational exposure scenario, in order to disclose whether the combined toxic effects of the mixture of Cr(VI), Nickel (Ni) and PAHs are different from the ones expected from the sum of its single components effect.

More specifically, it is intended to create an *in vitro* model to characterize the effect of chemical mixtures to which humans are exposed through inhalation. The combined cytotoxicity and genotoxicity of binary mixtures of Cr(VI) and Ni and of ternary mixtures including BaP will be determined and the results modelled.

3. Material and Methods

3.1. Cell culture

The A549 cell line (human epithelial lung cells, ATCC®) (figure 3.1) was first isolated in 1972 from a pulmonary adenocarcinoma and it is widely used in different studies where there is a need to mimic the lung environment. These cells possess molecules involved in cells detoxification. For this reason, the A549 cell line is a relevant model in the study of metabolic pathways and the mechanisms involved in drug and substance delivery and processing at the pulmonary epithelium. The doubling time of this cell line is approximately 22 hours (Giard *et al*., 1973).

The A549 were seeded and grown in RPMI medium supplemented with 1% Penicillin/Streptomycin, 1% fungizone and 10% inactivated fetal bovine serum (FBSi), at 37 ºC, in 5% CO_2 and humidified atmosphere. When the cells reached approximately 70% confluence, they needed to be subcultured. The medium was removed and the cells were washed and incubated with 2 mL trypsin-EDTA for 4 min at 37 ºC in order to detach the cells from the flask. Once detached, culture medium (RPMI) was added to the cell suspension in order to inactivate the trypsin and the cells were subcultured at previous chosen densities and maintained in the same conditions as described before. In these culture conditions, A549 cells reached confluence around 2 or 3 days. All reagents were provided by Thermo Fisher, Massachusetts, EUA.

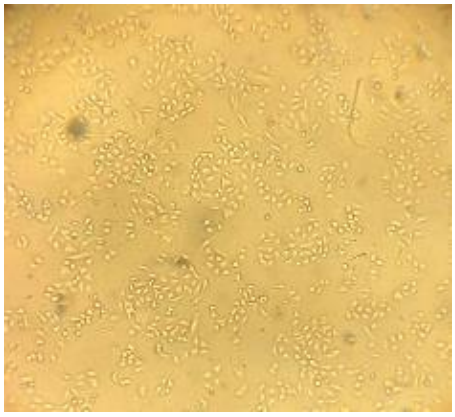


Figure 3.1 - A549 cells in RPMI medium.

3.2. Chemical solutions preparation

3.2.1. Hexavalent Chromium

Chromium(VI)oxide (CAS:1333-82-0) was obtained from Sigma Aldrich. Approximately 220 mg were weighted and posteriorly diluted in 50 mL of sterile bidistilled water in order to obtain a work solution with a final concentration of 44 mM. From this solution, treatment solutions, with different concentrations, were prepared in complete medium, as represented in Table 3.1.

Table 3.1 - Cr(VI) concentrations used for MTT and MN assay.

Cr(VI)		
MTT		MN
24h	**48h**	**24h**
0.625 µM	0.625 µM	0.625 µM
1.25 µM	1.25 µM	
2.5 µM	2.5 µM	1.25 µM
5 µM	5 µM	
7.5 µM		2.5 µM
10 µM		

3.2.2. Nickel

Nickel(II)Chloride hexahydrate (CAS:7791-20-0) was equally obtained from Sigma Aldrich and in this case, approximately 523 mg were weighted and diluted is 50 mL of sterile bidistilled water, obtaining a work solution of 44 mM. Treatment solutions, with different concentrations, were prepared in complete medium, as represented in Table 3.2.

Table 3.2 - Ni concentrations used for MTT and MN assay.

Ni		
MTT		MN
24h	**48h**	**24h**
0.25 mM	0.125 mM	0.25 mM
0.5 mM	0.25 mM	
0.75 mM	0.5 mM	
1 mM	0.75 mM	
1.25 mM	1 mM	0.5 mM
1.5 mM		
2 mM		

3.2.3. Benzo(a)pyrene

BaP (CAS: 50-32-8) was also obtained from Sigma Aldrich. In short, 19.82 mL of DMSO were added to the BaP flask, containing 100 mg of this compound, in order to obtain a work solution of 20 mM. From this solution, the different treatment solutions with different concentrations, were prepared in complete medium, as represented in Table 3.3. This compound was tested in the presence of a S9 fraction, in order to provide metabolic activation. Accordingly to the literature it should be used 1-2% of S9 in culture medium. Following previous protocols, we used 1.5% of S9. A positive control, with cyclophosphamide, for the S9 fraction was also added at a concentration of 10 µg/mL. Since BaP was reconstituted in DMSO, a vehicle control with DMSO (1%) was included. However, the maximum percentage of DMSO achieved in the culture medium was 0.5% for the highest BaP concentration tested in the MTT assay (100 µM) and 0.25% for highest BaP concentration tested in the MN assay (50 µM).

Table 3.3 - BaP concentrations used for MTT and MN assay.

BaP	
MTT	MN
24/48h	24h
0.1 µM	12.5 µM
1 µM	
10 µM	
12.5 µM	25 µM
25 µM	
50 µM	
75 µM	50 µM
100 µM	

3.2.4. Mixtures

For these assays, a range of concentrations of each compound was selected, based on the values of IC_{50} obtained and in data from the literature. After the cytotoxicity assessment, we selected the values near and bellow the IC_{50} for both genotoxicity assessment and cytotoxicity for the mixture's assays, as seen in Table 3.4 for binary mixtures and Table 3.5 for ternary mixtures.

Table 3.4 - Cr(VI) and Ni concentrations and combinations used for MTT and MN assay.

Binary mixture					
MTT				MN	
24h		**48h**		24h	
Cr(VI)	Ni	Cr(VI)	Ni	Cr(VI) + Ni	
0.625 µM	0.25 mM	0.625 µM	0.125 mM	0.625 µM	0.25 mM
0.625 µM	0.5 mM	0.625 µM	0.25 mM		0.5 mM
2.5 µM	0.25 mM	1.25 µM	0.125 mM	1.25 µM	0.25 mM
2.5 µM	0.5 mM	1.25 µM	0.25 mM		0.5 mM
				2.5 µM	0.25 mM
					0.5 mM

Table 3.5 - Cr(VI), Ni and BaP concentrations and combinations used for MTT and MN assay.

Ternary mixture		
MTT 24/48h and MN 24h		
Cr(VI)	Ni	BaP
0.625 µM	0.25 Mm	12.5 µM

3.3. Cytotoxicity Assessment

3.3.1. MTT Assay

For this assay, A549 cell line was seeded at a density of $0{,}5x10^4$ cells per well in a 96-well plate and incubated at 37ºC, 5% CO_2, for 24h. Then, the cells were exposed during 24 and 48h to the treatment solutions of each compound, previously prepared at different concentrations, 6 wells per treatment condition. The negative control includes cells without the treatment solutions, and for the positive control we used SDS solution at 0.1% which promotes cell lysis. This solution was added to the cells 1h before adding the MTT solution (t=23h/t=43h). After the exposure time, the treatment medium was removed and the wells were washed twice with PBS 1x (Thermo Fisher, Massachusetts, EUA) and then added 100 µL of MTT solution to each well and incubated for 3h at the previous conditions. The MTT solution was previously prepared in cell culture medium and PBS 1x, at a final concentration of 0.5 mg/mL. After 3h of incubation, the MTT solution was removed and 100 µL of dimethyl sulfoxide (DMSO) (Sigma Aldrich) was added to each well followed by a 30 min period of agitation in order to dissolve the crystals formed. In the end, the absorbance of each well was measured in a Multiskan Ascent Spectrophotometer (Thermo LabSystems, Waltham, MA) at 570 nm (reference filter: 690 nm). Three independent assays were performed for each compound and mixture of compounds.

3.4. Genotoxicity Assessment

3.4.1. Cytokinesis-blocked micronucleus assay (CBMN)

For this assay, performed following the OECD guideline 487, the A549 cell line was seeded at a density of $2x10^5$ cells per well in a 6-well plate and incubated at 37ºC, 5% CO_2, for 24h. After 24 h, the cells were exposed during 24h to the treatment solutions of each compound tested, previously prepared at different concentrations, and incubated at 37ºC, 5% CO_2. At t=23h Mitomycin C (MMC) was added, as a positive control, at 0.01 µg/µL. Once again, the negative control includes cells with culture medium, without the treatment solutions. After adding the positive control, at t=24h after exposure, the treatment was removed and the wells were washed twice with PBS 1x and fresh medium with cytochalasin B at 6 µg/mL was added to the wells, including positive and negative controls. The cells were then incubated at 37ºC, 5% CO_2 for 42h. After this period of time, the cells were recovered from the plates, the medium was removed and the wells were washed with PBS x1 and trypsine EDTA (0.05%) was added to each well, in order to detach the cells from the wells. The cell suspension was then transferred to a 15 mL centrifuge tube and were centrifuged at 1000 rpm for 5 minutes. After this process the supernatant was discarded and the cell pellet was resuspended. A volume of 5 mL of the hypotonic solution (73.5% of dd sterile water; 24.5% of RPMI medium; 2% of FBSi) was added to each tube, drop by drop, while vortexing the tube. The tubes were immediately centrifuged, at 1000 rpm for 5 minutes, after the solution was added. The supernatant was then discarded, the pellet was resuspended and drops of 20 µL were spread on the slides. After this process the slides were allowed to dry and then were fixed in cold methanol for 20 minutes. For the staining process, the cells were immersed in a 4% Giemsa solution (Merck, Darmstadt, Germany) diluted in Gurr's phosphate buffer, previously prepared, for 15 minutes and finally washed twice with the same buffer. The slides were then allowed to dry and mounted with Entellan® (Merck, Darmstadt, Germany) and cover slips.

The slides were coded and analyzed under an optical microscope (Axioskop 2 Plus, Zeiss, Germany) and micronuclei were scored. 500 binucleated cells were analyzed per slide, that is, 1000 cells per culture and 2000 cells per treatment. Micronuclei diameter must be less than 1/3 of the diameter of the main nuclei, it also must have a round or oval shape and be separated from the main nuclei. (Fenech *et al*., 2003) (figure 3.2). Only one independent assay, with 2 replicates, was performed for each

substance and for the mixtures tested. The used criteria for scoring micronucleus are described in Fenech *et al*., 2003.

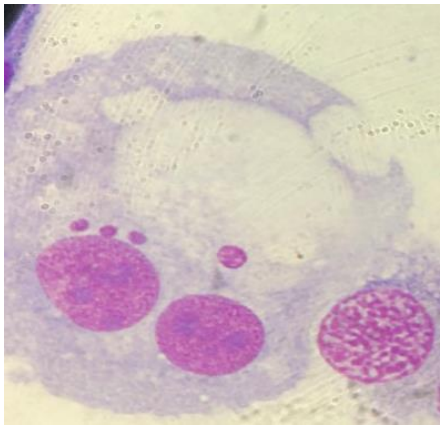


Figure 3.2 - Micronucleus in A549 cells exposed to Cr(VI) (amplification 10x100).

Based on the number of mononucleated, binucleated and multinucleated scored in a total of 1000 cells for each treatment, the cytokinesis-blocked proliferation index (CBPI) and replication index (RI) were calculated. CBPI indicates the average number of cell cycles per cell, during the period of exposure to cytochalasin B. RI indicates the relative number of nuclei in the treated cultures, compared to the control cultures, in percentage. Alterations in both indices may indicate cytotoxic effects from an agent. CBPI and RI were calculated through the following equations (OECD, 2016):

$$\text{CBPI} = \frac{(\text{nrº mononucleated cells}) + (2 \times \text{nrº binucleated cells}) + (3 \times \text{nrº multinucleated cells})}{\text{Total number of cells}}$$

$$\text{RI} = \frac{\left(\frac{\text{nrº binucleated cells} + 2 \times \text{nrº multinucleated cells}}{\text{Total number of cells}}\right) \text{Treated cultures}}{\left(\frac{\text{nrº binucleated cells} + 2 \times \text{nrº multinucleated cells}}{\text{Total number of cells}}\right) \text{Control cultures}}$$

3.5. Statistical analysis

Statistical analysis was performed using the IBP SPSS Statistics 26 program. In all testes a significance level of 0.05 was considered. To analyze the results of the cytotoxicity test, MTT, the ANOVA test and the respective Post-Hoc test were used. Tuckey Post-Hoc was used when homoscedasticity was verified, and Dunnet T3 Post-Hoc was used when homoscedasticity wasn't verified. To analyze the results of the micronucleus assay, Fisher's exact test was performed to compare the frequency of binucleated cells with micronuclei between cultures exposed to the chemicals and the control cultures. A regression analysis was performed in Excel to verify the existence of dose-response relationship. To analyze CBPI and RI, no statistical analysis was performed since only one experimental replica was performed.

Data obtained from the mixtures exposures was analyzed using the program MIXTOX by comparing the data with the predicted effects, and using the reference models (CA and IA). Both models were mathematically extended to derive deviations for synergism/antagonism, dose ratio and dose level dependency, by forming a nested framework. From this mathematical modeling, there were two output parameters (a and b) that enable the prediction of a biological effect. When a significant deviation from CA and IA model was identified, the effect pattern was deduced directly from the parameter values (figure 3.3).

Parameter	Value		Meaning
	CA	IA	
		Synergism/antagonism	
a	>0	>0	Antagonism
	<0	<0	Synergism
		Dose ratio dependence	
a	>0	>0	Antagonism, except for those mixture ratios where significant negative b_is indicate synergism
	<0	<0	Synergism, except for those mixture ratios where significant positive b_is indicate antagonism
b_i	>0	>0	Antagonism where the toxicity of the mixture is caused mainly by toxicant i
	<0	<0	Synergism where the toxicity of the mixture is caused mainly by toxicant i
		Dose level dependence	
a	>0	>0	Antagonism low dose level and synergism high dose level
	<0	<0	Synergism low dose level and antagonism high dose level
b_{DL}	>1	>2	Change at lower dose level than the EC50
	$=1$	$=2$	Change at the EC50 level
	$0 < b_{DL} < 1$	$1 < b_{DL} < 2$	Change at higher dose level than the EC50
	<0	<1	No change, but the magnitude of synergism/antagonism is dose level (CA) or effect level (IA) dependent

[a] EC50 = median effect concentration.

Figure 3.3 - Analysis of mixture toxicity data and interpretation of parameters a and b that define the functional formo f deviation pattern from the reference models of CA and IA. Adapted from Jonker *et al.*, 2009.

4. RESULTS

4.1. Cytotoxicity assessment

4.1.1. Single substances

4.1.1.1. Hexavalent Chromium (Cr(VI))

Concerning the cytotoxicity results 24h after exposure (figure 4.1a), all Cr(VI) concentrations, except the lowest one (0.625 µM), have a significant effect on cell viability, when compared with the negative control ($p \leq 0.002$, ANOVA, Tukey Post-Hoc). After 48h of exposure (figure 4.1b), only the two highest concentrations tested (2.5 and 5 µM), induce a significant effect on cell viability ($p \leq 0.001$, ANOVA, Dunnett's T3 Post-Hoc).

After adjusting the curves through regression analysis, a concentration-effect relationship is observed, for both exposure times. The equation that better describes the 24h curve is: $y=-0.9489x^2+16.689x+5.3941$, from which an IC_{50} value of 3.30 µM was derived. Concerning 48h exposure, the equation is: $y=-56855x^2+47.257x-1.154$, from which an IC_{50} value of 1.28 µM was derived. The positive control (SDS) induced 91.7 and 95.4% relative cytotoxicity for 24 and 48h, respectively, which is significantly different from the negative control ($p<0.001$).

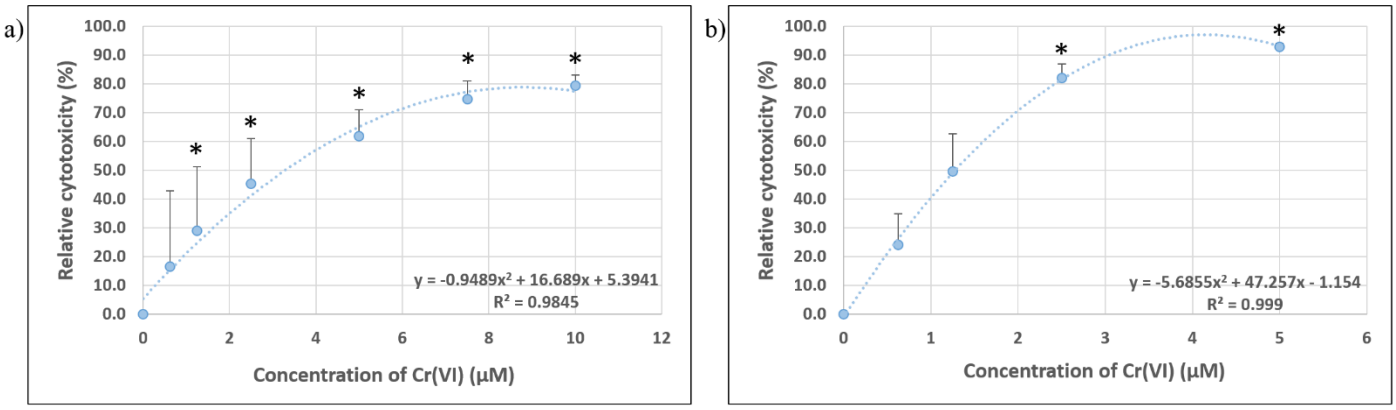


Figure 4.1 - Cytotoxicity results (MTT assay) in A549 cells exposed to Cr(VI), a) 24h of exposure; b) 48h of exposure. Positive control (SDS) produced 91.7% and 95.4% relative cytotoxicity for 24 and 48h, respectively. * indicates significant differences from the negative control.

4.1.1.2. Nickel

At 24h exposure time (figure 4.2a), nickel increased significantly cell lethality in all tested concentrations, when compared with the negative control ($p=0.038$; $p=0.008$; $p=0.006$; $p \leq 0.002$ for 0.25 mM, 0.5 mM 0.75 mM, 1 mM, 1.25 mM, 1.5 mM and 2 mM, respectively, ANOVA, Dunnett's T3 Post-Hoc). At 48h exposure time (figure 4.2b), all concentrations tested, except the lowest one (0.125 mM), show a significant effect on cell viability ($p \leq 0.002$, ANOVA, Dunnett's T3 Post Hoc).

After adjusting the curve through regression analysis, a concentration-effect relationship is observed, for both exposure times. The equation that better describes the 24h curve is: $y=-37.812x^2+109.36x+4.3231$, from which an IC_{50} value of 0.51 mM was derived. Concerning 48h exposure, the equation is: $y=-151.4x^2+239.99x+4.494$, from an IC_{50} value of 0.22 mM was derived. Positive control (SDS) induced 94.7 and 96.9% relative cytotoxicity for 24 and 48h, respectively, which is significantly different from the negative control ($p<0.001$).

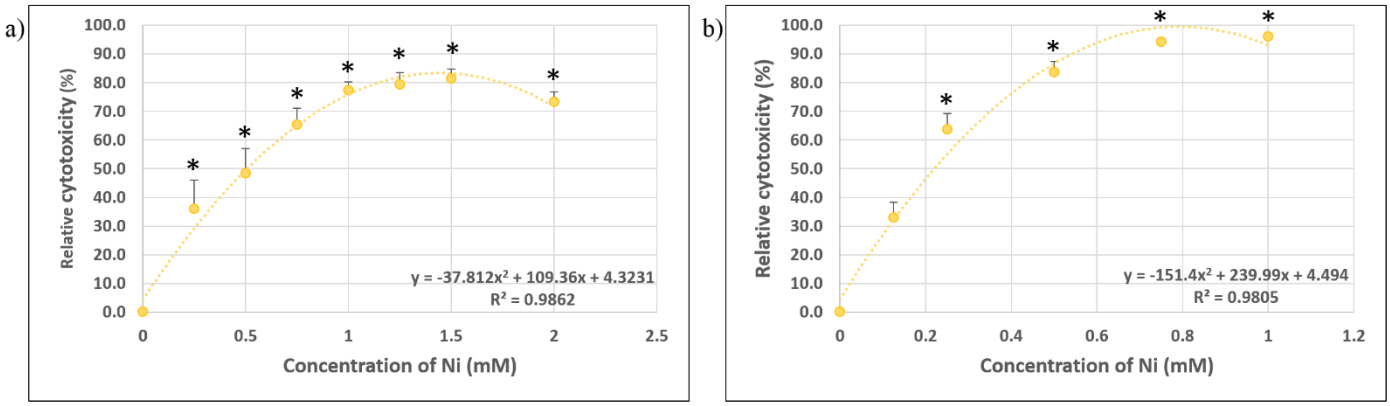


Figure 4.2 - Cytotoxicity results (MTT assay) in A549 cells exposed to Ni, a) 24h of exposure; b) 48h of exposure. Positive control had 94.7% and 96.9% relative cytotoxicity. * indicates significant differences from the negative control.

4.1.1.3. Benzo(a)pyrene (BaP)

Regarding BaP cytotoxicity, after 24h of exposure (figure 4.3a), only one concentration (100 µM) caused a significant cell death when compared with the negative control ($p=0.039$, ANOVA, Dunnett's T3 Post-Hoc). The vehicle control with DMSO (1%), showed significant differences when compared with the negative control, with 23.65% relative cytotoxicity ($p=0.016$). However, given that DMSO top concentration achieved in culture was 0.5%, this value was not considered for calculating the relative toxicity of BaP. The positive control (SDS) had 93.1% relative cytotoxicity, significantly different from the negative control ($p<0.001$).

For 48h exposure (figure 4.3b), once again, no significant decrease in cells viability was observed, except for the concentration of 100 µM (p=0.010, ANOVA, Tukey Post-Hoc). DMSO control also showed significant differences when compared with the negative control, with 29.49% relative cytotoxicity (p=0.019), and the positive control with SDS also showed significant differences, with 95.7% relative cytotoxicity. In this assay, for both exposure times, we couldn't reach an IC_{50} value due to the low cytotoxicity of BaP. The data didn't allow to define a concentration-response relationship and no satisfactory curve equation was found.

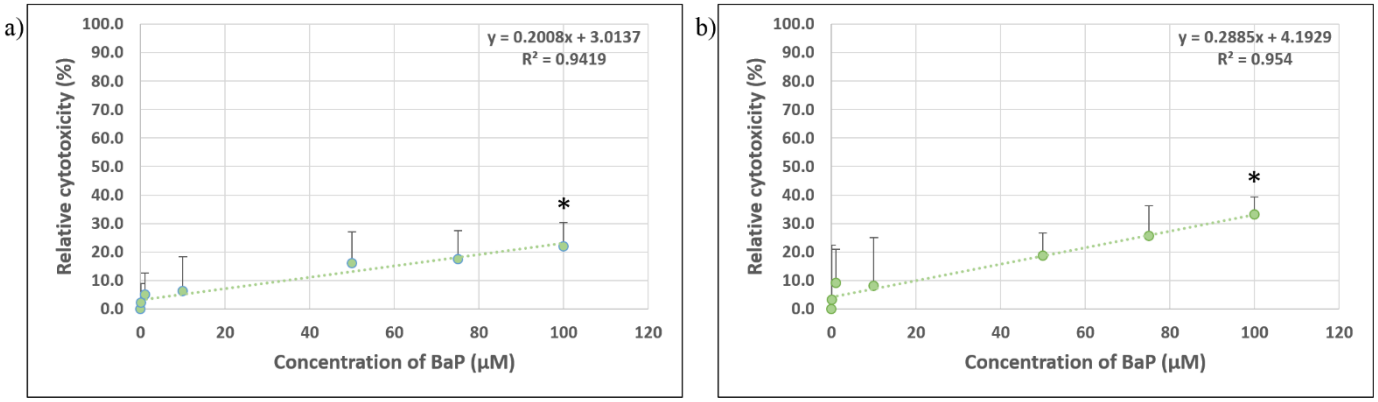


Figure 4.3 - Cytotoxicity results (MTT assay) in A549 cells exposed to BaP, a) 24h of exposure; b) 48h of exposure. DMSO control had 23.65% and 29.49% relative cytotoxicity for 24 and 48h, respectively and positive control (SDS) had 93.1% and 95.7% relative cytotoxicity for 24 and 48h, respectively. * indicates significant differences from the negative control.

A S9 fraction was then added to cells culture to provide metabolic activation of BaP. Still, no significant differences in BaP toxicity were found (p>0.05) and also no evident differences were observed when comparing with the treatment without S9 fraction for both exposure times (24 and 48h) (figure 4.4a, 4.4b). In these cases, since we used S9 fraction, we also used the specific positive control, cyclophosphamide that didn't show significant differences when compared with the negative control, with 11.13% and 13% relative cytotoxicity for 24 (p=0.778) and 48h (p=0.271), respectively. Positive control (SDS) had 92.5 and 94.7% relative cytotoxicity for 24 and 48h, respectively, which is significantly different from the negative control (p<0.001).

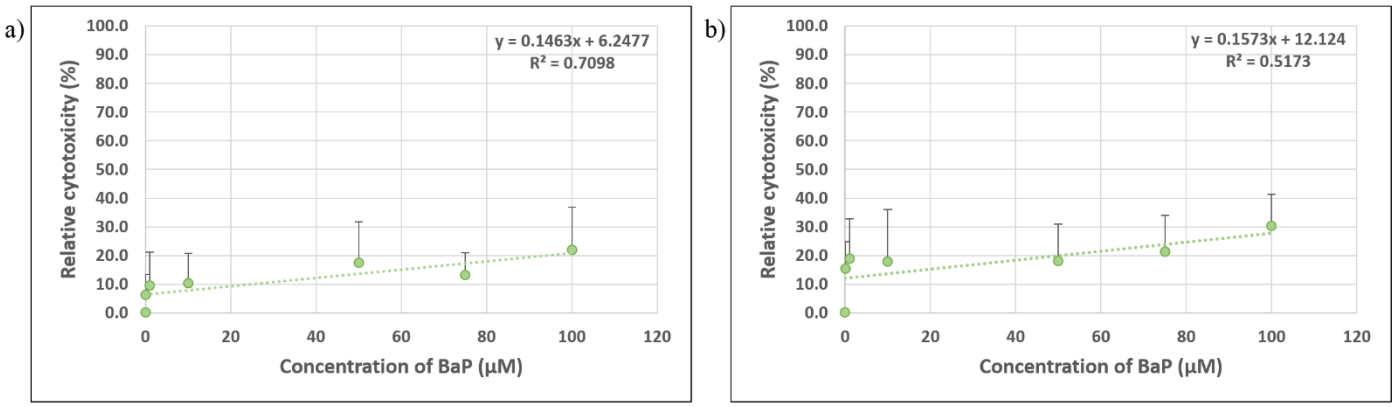


Figure 4.4 - Cytotoxicity results (MTT assay) in A549 cells exposed to BaP with S9 fraction, a) 24h of exposure; b) 48h of exposure. DMSO control had 27.88% and 33.86% relative cytotoxicity for 24 and 48h, respectively. Cyclophosphamide control had 11.13% and 13% relative cytotoxicity for 24 and 48h, respectively, and positive control (SDS) had 92.5% and 94.7% relative cytotoxicity for 24 and 48h, respectively. * indicates significant differences from the negative control.

4.1.2. Mixtures

4.1.2.1. Binary Mixtures

For both 24 and 48h of exposure, all different concentrations of single substances or their combinations significantly increased cytotoxicity (figure 4.5a, 4.5b) (p<0.01, ANOVA, Tukey Post-Hoc). It can be observed that cytotoxicity increases with the increase of the components concentrations in the mixture, reaching the highest level when we have in mixture the two highest concentrations of Cr(VI) and Nickel (2.5 µM and 0.5 mM, respectively). The positive control (SDS) for both exposure

times had 94.2 and 96.2% relative cytotoxicity for 24 and 48h, respectively, which is statistically different from the negative control (p<0.001).

For 24h exposure the only mixture that yielded statistically significant different cytotoxicity from its individual components is the combination of 2.5 µM Cr(VI) (p<0.001) and 0.25 mM Ni (p=0.004). The other three mixtures (0.625 µM Cr(VI) + 0.25 mM Ni; 0.625 µM Cr(VI) + 0.5 and 2.5 µM Cr(VI) + 0.5 mM Ni) produced levels of cell death that were significantly different from those produced by the respective concentrations of Cr(VI) (p<0.001 for all three combinations), but not from the nickel concentrations.

After 48h of exposure the results of the mixtures with 1.25 µM Cr(VI) (p=0.003) + 0.125 mM Ni (p≤0.001) and 1.25 µM Cr(VI) (p<0.001) + 0.25 mM Ni (p≤0.03), are significantly different from their individual components in the combination. The results of the other two mixtures (0.625 µM Cr(VI) + 0.125 mM Ni and 0.625 µM Cr(VI) + 0.25 mM Ni) were significantly different from the concentrations of Cr(VI) (p<0.001 for both combinations), but not from the nickel concentrations.

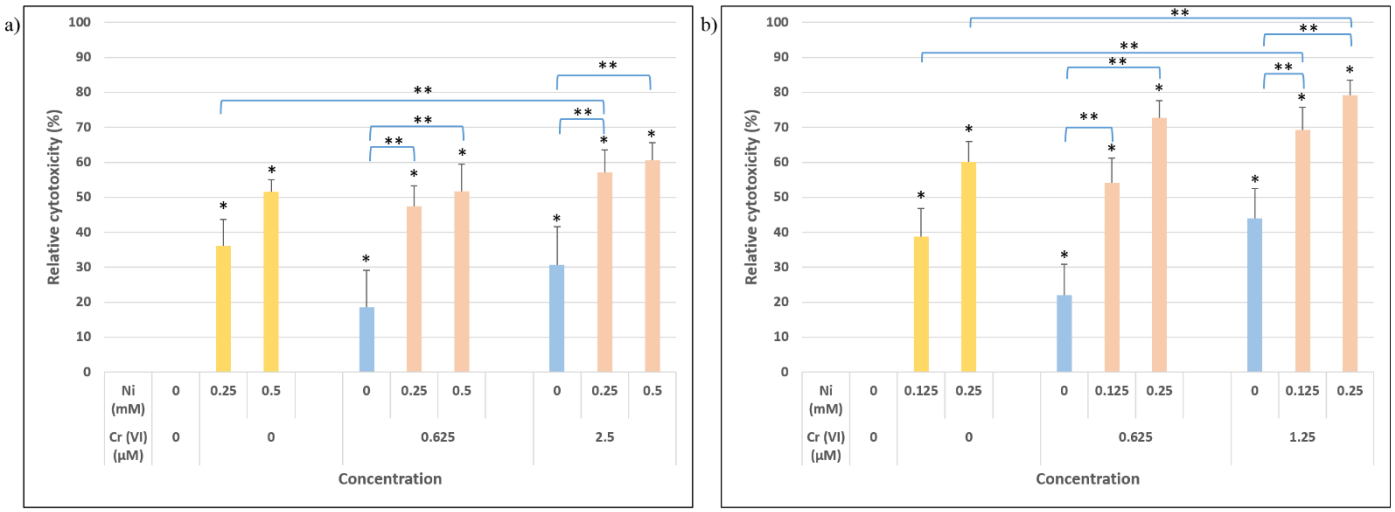


Figure 4.5 - Cytotoxicity results (MTT assay) in A549 cells exposed to Ni and Cr(VI) and binary mixtures of these substances, a) 24h of exposure; b) 48h of exposure. Positive control (SDS) had 94.2% and 96.2% relative cytotoxicity for 24 and 48h, respectively. * indicates significant differences from the negative control. ** indicates significant differences between the mixture and corresponding single substance.

A mathematical tool was used to explore the existence of additivity or deviations from that effect towards a synergism or antagonism. The modelling results showed that the combined effect observed was different depending on whether we use the CA or the IA model. For both 24 and 48h, using the CA model (figure 4.6a; 4.7a), a weak antagonistic effect was found (a=0.57 for 24h and a=0.48 for 48h, considering figure 3.3); using the IA model (figure 4.6b; 4.7b), a synergistic effect was found for lower concentrations, with an alteration to antagonism at the concentrations above the IC_{50} of the mixture (a=-3,23; b_{DL}=2.01 for 24h and a=-2.45; b_{DL}=1.33 for 48h, considering figure 3.3).

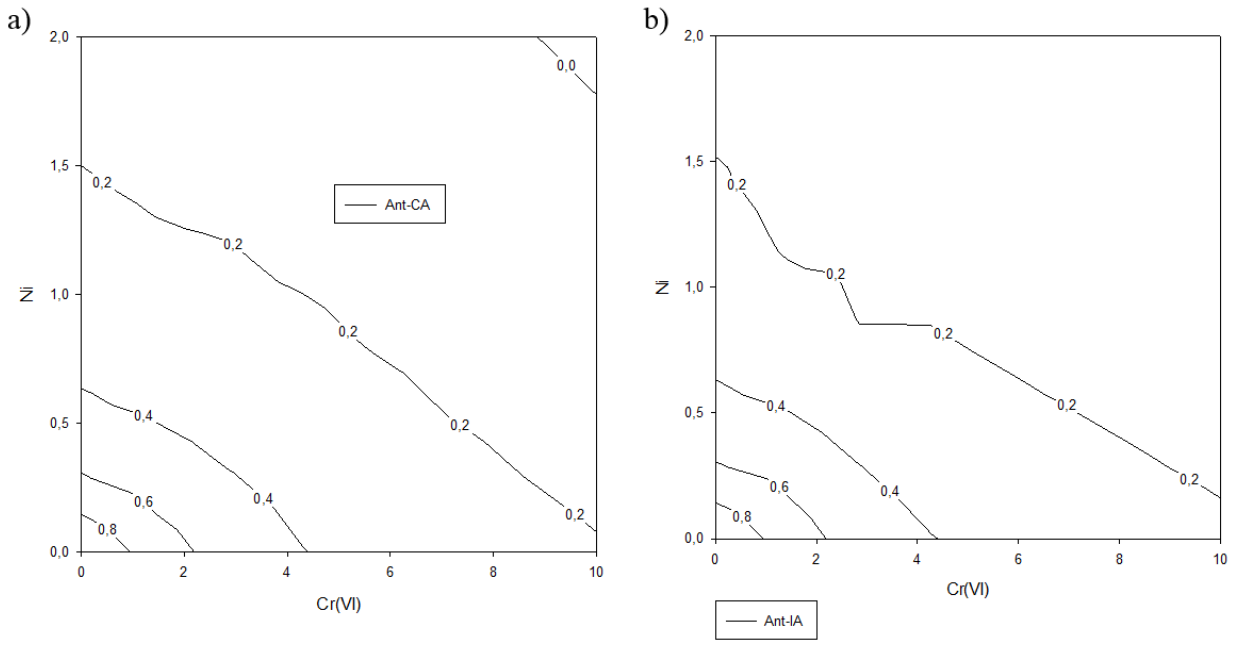


Figure 4.6 - Isobologram for modeling the citotoxic effects of Cr(VI) and Ni mixtures in A549 cells, after 24h of exposure, a) CA model; b) IA model

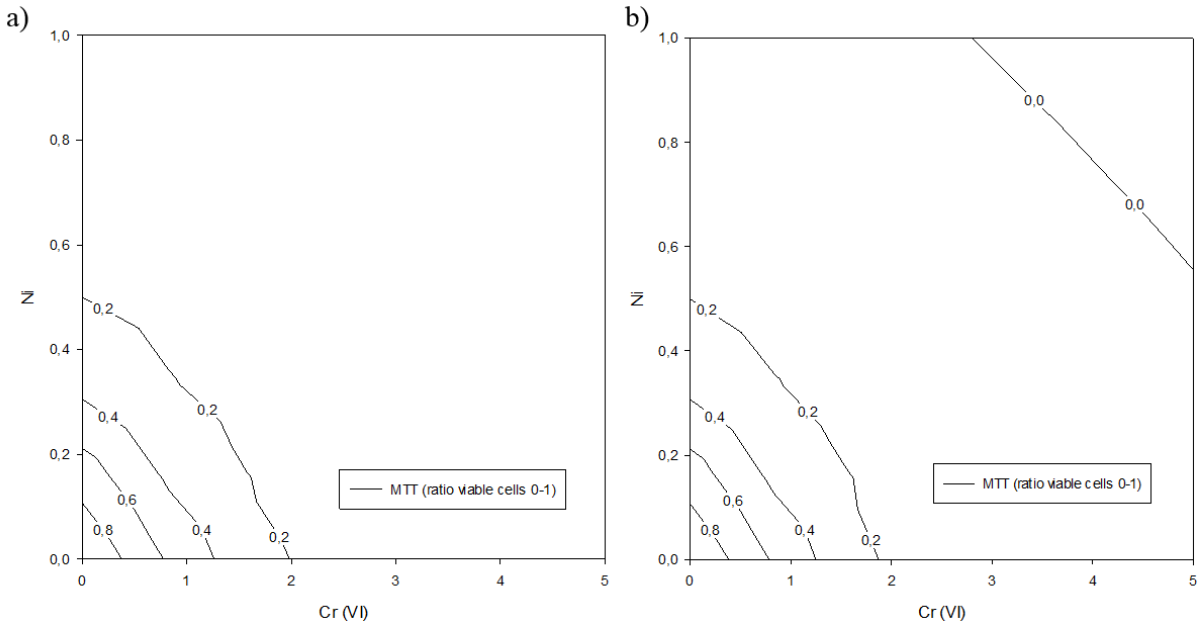


Figure 4.7 - Isobologram for modeling the citotoxic effects of Cr(VI) and Ni mixtures in A549 cells, after 48h of exposure, a) CA model; b) IA model

4.1.2.2. Ternary Mixtures

At 24h of exposure, both the individual substances and their combinations caused a significant increase in cell death when compared with the negative control (figure 4.8a) (p=0.05, p=0.005, p=0.002, for 12.5 µM BaP, 0.25 mM Ni and 0.625 µM Cr(VI), respectively, and p=0.014 for BaP + Ni + Cr(VI), ANOVA, Dunnett's T3 Pos-Hoc). It can be observed that the relative cytotoxicity is higher for the mixture of the three substances than for the individual components, even though this difference isn't statistically significant (p>0.05). Given that only a ternary mixture was tested due to time constrains, the data generated was insufficient for modeling. Nevertheless, from the results obtained we can empirically infer that the combined toxicity of the 3 substances was lower than expected from the sum of their toxicity of the single substances, resembling antagonism.

After 48h of exposure (figure 4.8b), only the concentration of 12.5 µM BaP didn't induce a significant increase in cell death (p=0.270). Once again, the relative cytotoxicity is higher in the mixture, and in this case, this mixture toxicity is statistically different from that obtained for two of its components, 12.5 µM BaP (p<0.001) and 0.625 µM Cr(VI) (p=0.011). Similarly to the mixture effect obtained with a 24h exposure, also a longer exposure to the ternary mixture generated a lower than additive toxic effect in A549 cells.

The positive control (SDS) had 94 and 96.6% relative cytotoxicity for 24 and 48h, which is significantly different from the negative control (p<0.001).

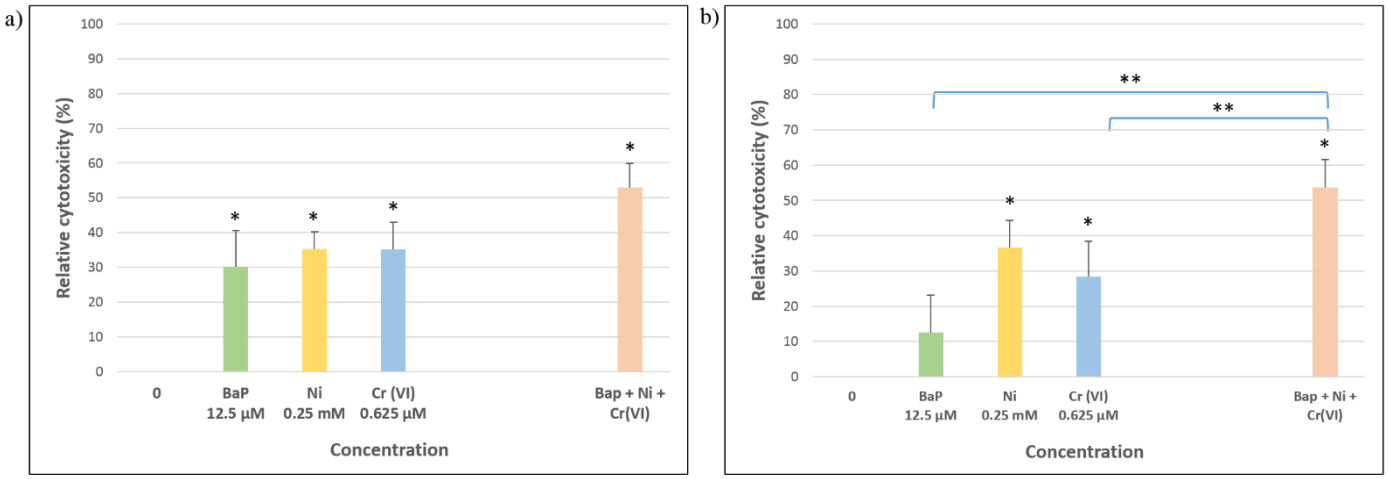


Figure 4.8 - Cytotoxicity results (MTT assay) in A549 cells exposed to BaP, Ni, Cr(VI) and a ternary mixture of these compounds, a) 24h of exposure; b) 48h of exposure. DMSO control had 29.43% and 20.90% relative cytotoxicity, for 24 and 48h, respectively and positive control (SDS) had 94% and 96.6% relative cytotoxicity for 24 and 48h, respectively. * indicates significant differences from the negative control. ** indicates significant differences between the mixture and corresponding single substance.

4.2. Genotoxicity assessment

4.2.1. Single substances

4.2.1.1. Hexavalent Chromium (Cr(VI))

After 24h of exposure (figure 4.9), all three concentrations tested with the micronucleus assay induced significant increases in micronucleus frequencies when compared with the negative control (p≤0.001, Fisher's Exact Test). A concentration-dependent effect was observed, as the frequency of micronuclei increases, with the tested concentrations. Positive control (MMC) also increased significantly the frequency of micronuclei when compared with the negative control (p<0.001), presenting a value of 29 micronucleated cells per 1000 binucleated cells.

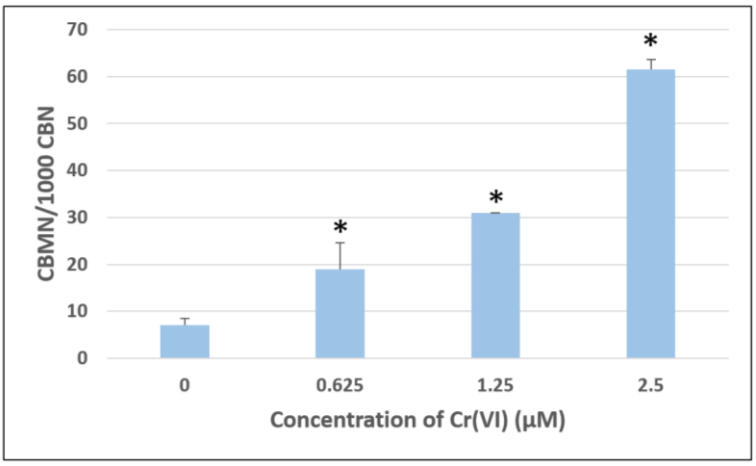
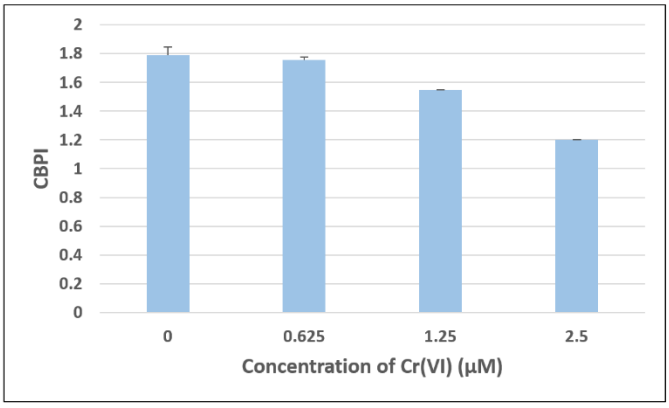


Figure 4.9 - Genotoxicity results (MN assay) in A549 cells exposed to Cr(VI) for 24h. Positive control (MMC) had a value of 29 micronucleated cells per 1000 binucleated cells. * indicates significant differences from the negative control.

We can observe that CBPI and RI both decrease, with the increase of Cr(VI) concentrations, showing also a concentration-dependent effect on the cell cycle progression and the cells' capacity to divide. (figure 4.10a, 4.10b).

a)



b)

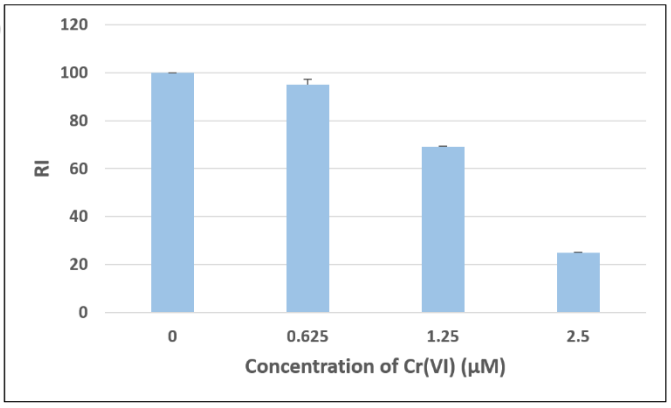


Figure 4.10 - a) CBPI and b) RI results in A549 cells exposed to Cr(VI) for 24h.

4.2.1.2. Nickel

Concerning the genotoxicity of Ni, both Ni concentrations significantly increased the frequency of micronuclei when compared with the negative control, after 24h of exposure (figure 4.11) (p=0.002; p<0.001 for 0.25 mM and 0.5 mM, respectively, Fisher's Exact Test); although only two concentrations were tested, a concentration-dependent effect was observed. When compared with the negative control, positive control (MMC) increased significantly the frequency of micronuclei (p<0.00), with 20 micronucleated cells per 1000 binucleated cells.

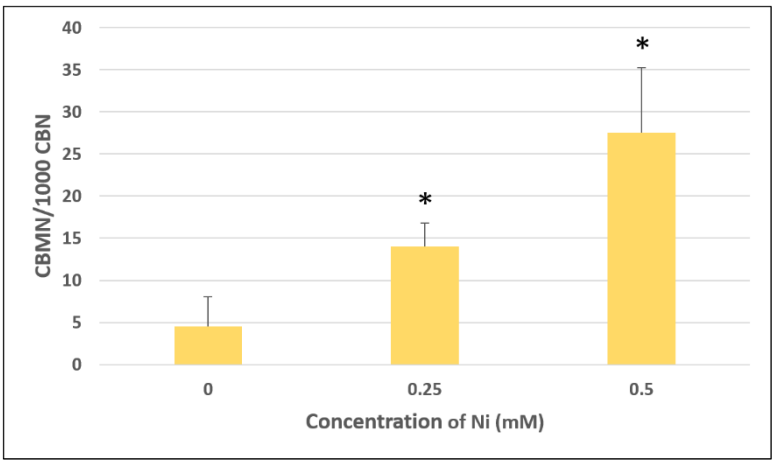


Figure 4.11 - Genotoxicity results (MN assay) in A549 cells exposed to Ni for 24h. Positive control (MMC) had a value of 20 micronucleated cells per 1000 binucleated cells. * indicates significant differences from the negative control.

We can observe that CBPI and RI both decrease, with the increase of the tested Ni concentrations, showing Ni effects at cell cycle progression and cells replicative potential levels (figure 4.12a, 4.12b).

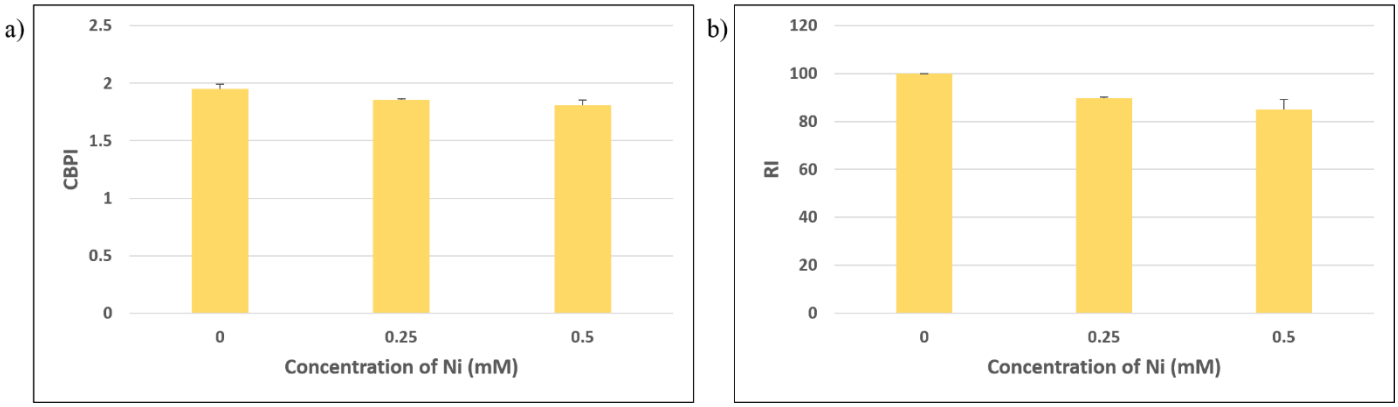


Figure 4.12 - a) CBPI and b) RI results in A549 cells exposed to Ni for 24h.

4.2.1.3. Benzo(a)pyrene

After 24h of A549 cells exposure, all BaP concentrations resulted in a significant increase of the frequency of micronuclei in relation to the negative control (Figure 4.13) (p=0.010; p=0.004; p=0.010 for 12.5, 25 and 50 µM, respectively, Fisher's Exact Test).The positive control with MMC showed significant differences from the negative control (p<0.001), with 21 micronucleated cells per 1000 binucleated cells.

With the addition of the exogenous metabolic system (S9 fraction), all concentrations showed also significant differences in the micronuclei frequencies compared with the negative control ($p\leq$0.001, Fisher's Exact Test). With the cyclophosphamide control there were also significant differences, with 11 micronucleated cells per 1000 binucleated cells (p=0.001). Positive control (MMC) showed a significant increase in the MN frequency over the negative control (p<0.001), presenting a value of 22 micronucleated cells per 1000 binucleated cells.

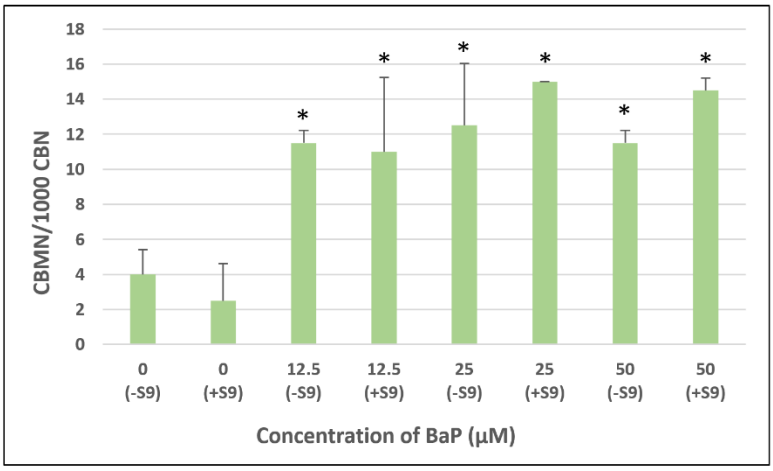


Figure 4.13 - Genotoxicity results (MN assay) in A549 cells exposed to BaP, with and without S9 fraction, for 24h. DMSO control had a value of 4 and 9 micronucleated cells per 1000 binucleated cells and positive control (MMC) had a value of 21 and 22 micronucleated cells per 1000 binucleated cells, for BaP exposure with and without S9 fraction, respectively. Cyclophosphamide control had a value of 11 micronucleated cells per 1000 binucleated cells. * indicates significant differences from the negative control.

The 12.5 and 25 µM concentrations were used based on available literature. Since BaP didn't prove to be cytotoxic, we decided to explore different concentrations for the micronucleus assay. However a cytotoxicity assessment for those two concentrations was also performed with 7.6% and 25.3% relative cytotoxicity for 12.5 and 25 µM, respectively, for 24h exposure time. For 48h exposure, 12.5 and 25 µM concentrations presented a value of 14.4 and 25.6% relative cytotoxicity, respectively (not represented graphically).

No effects of BaP were observed on the CBPI and RI (figure 4.14a, 4.14b).

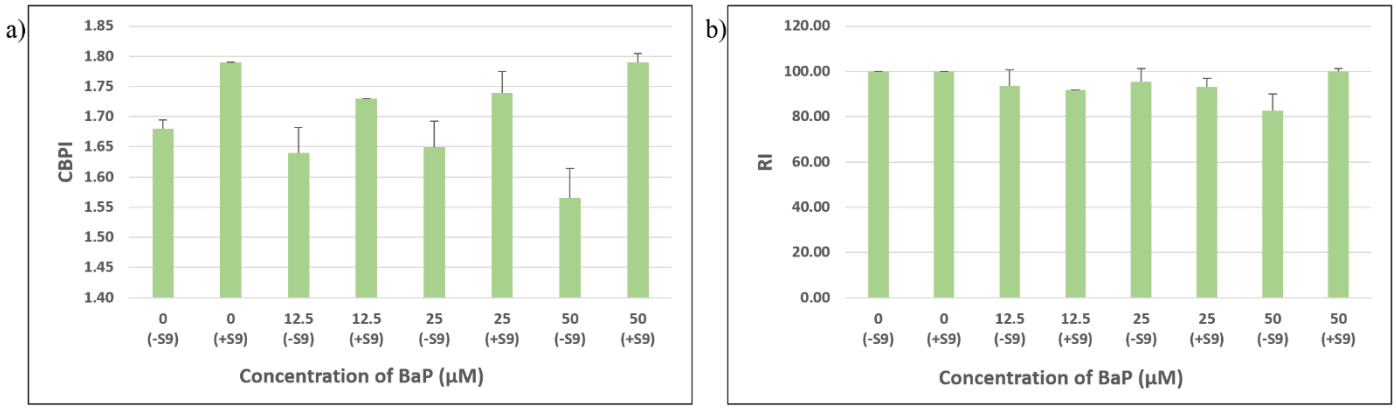


Figure 4.14 - a) CBPI and b) RI results in A549 cells exposed to BaP, with and without S9 fraction for 24h.

4.2.2. Mixtures

4.2.2.1. Binary Mixtures

Concerning the genotoxicity assessment, after 24h exposure, all different concentrations of the single substances and the binary combinations tested, significantly increased the frequency of micronuclei when compared with the negative control (figure 4.15) ($p \leq 0.038$, Fisher's Exact Test). Micronuclei frequencies increased with the increase of the concentrations in the mixture, reaching the highest level with the combination of 1.25 µM Cr(VI) + 0.5 mM Ni. Although two more combinations of Ni and Cr(VI) were tested (2.5 µM Cr(VI) + 0.25 mM Ni and 2.5 µM Cr(VI) + 0.5 mM Ni), few cells remained to be scored when observed at the microscope. Therefore, it was impossible to quantify the micronucleus frequency, as well as to determine CBPI and RI. The results generated by all mixtures analyzed were significantly increased compared with the results obtained for the individual components ($p \leq 0.01$ for all combinations). Positive control (MMC) showed significant differences ($p<0.001$), presenting a value of 42 micronucleated cells per 1000 binucleated cells.

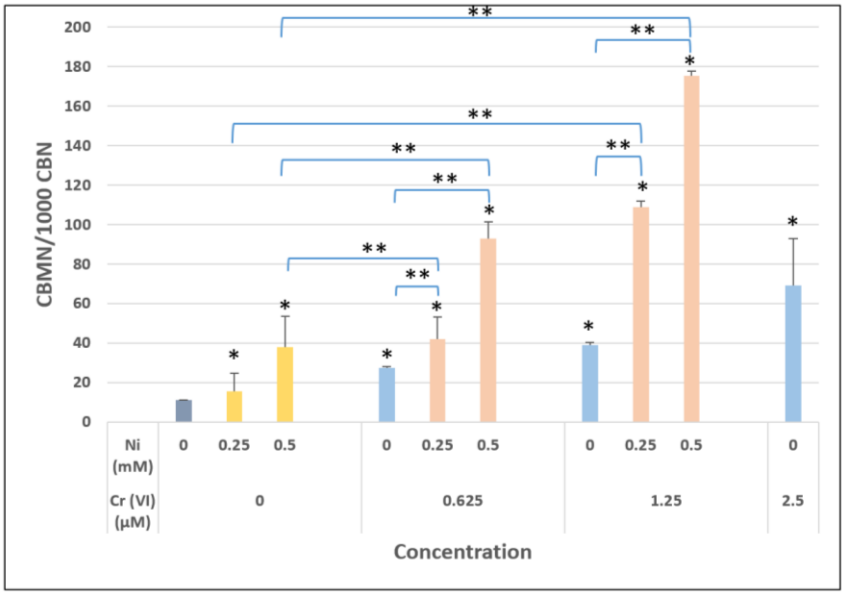


Figure 4.15 - Genotoxicity results (MN assay) in A549 cells exposed to Ni and Cr(VI) and binary mixtures of these compounds, for 24h. Positive control (MMC) had a value of 42 micronucleated cells per 1000 binucleated cells. * indicates significant differences from the negative control. ** indicates significant differences between the mixture and corresponding single substances.

The results of mathematical modelling showed that using the CA model, an additivity effect was found; using the IA model, a synergistic effect (a=-1.99, considering figure 3.3) was found (figure 4.16a; 4.16b).

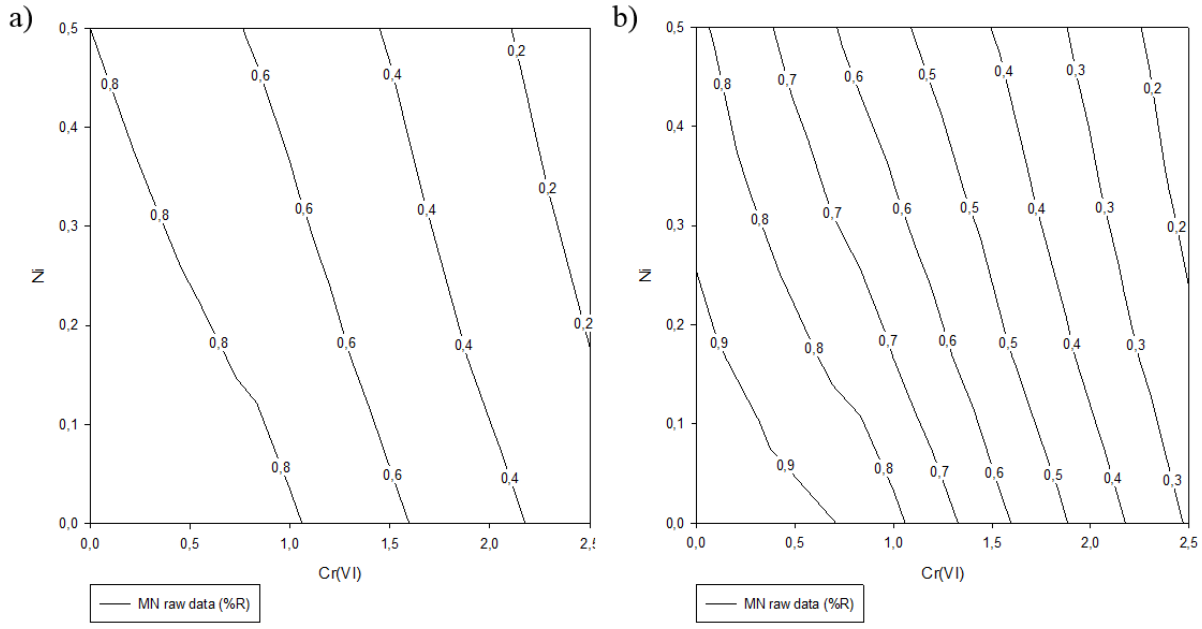


Figure 4.16 - Isobologram for modeling the genotoxic effects of Cr(VI) and Ni mixtures in A549 cells, after 24h of exposure, a) CA model; b) IA model

CBPI mainly decreases with the increase of individual Cr(VI) concentrations and with the increase of the concentrations in mixture, showing effects at cell cycle level (figure 4.17a, 4.17b). As referred above, we couldn't analyze the two mixtures with 2.5 µM of Cr(VI), possibly because they impaired cells division.

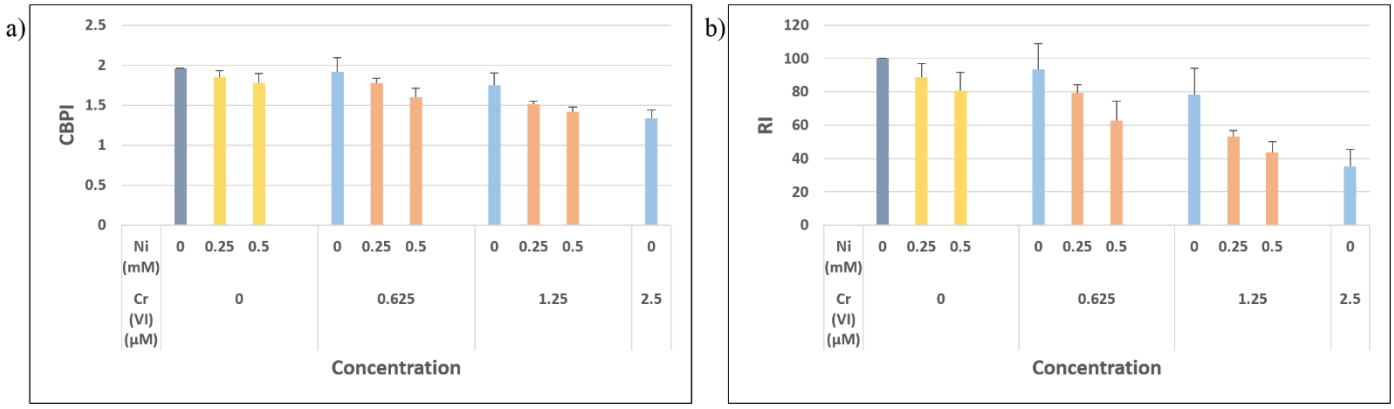


Figure 4.17 - a) CBPI and b) RI results in A549 cells exposed to Ni and Cr(VI) and binary mixtures of these compounds, for 24h.

4.2.2.2. Ternary Mixtures

All different concentrations and combinations, except 12.5 µM BaP, induced significant increases in the frequency of micronuclei when compared with the negative control (p=0.016, p<0.001, for 0.25 mM Ni and 0.625 µM Cr(VI), respectively, and p<0.001 for BaP + Ni + Cr(VI), Fisher's Exact Test) (figure 4.18). The combination between the 3 substances produced a higher micronucleus frequency than the individual components concentrations. This mixture is only statistically different from the concentration of 12.5 µM BaP (p<0.001) and 0.25 mM Ni (p=0.001), not from the Cr(VI) concentration. Given that we have the results from a single ternary mixture, it is not possible to have an adequate mathematical modelling, as previously referred, only a rough consideration about the mixture

effect can be done. Considering that each chemical per se is able to induce micronuclei, if their effects were additive, we would expect to observe a very high MN frequency, which was not the case. Opposite to that assumption, the frequency of micronucleated cells induced by the mixture was similar to the one induced by Cr(VI) alone. These observations suggest an antagonistic effect. MMC positive control was also statistically different, presenting a value of 40 micronucleated cells per 1000 binucleated cells (p<0.001).

In this case, after adding the S9 fraction, all different concentrations and combinations had significant differences in the frequency of micronuclei (p=0.029, p=0.016, p<0.001, for 12.5 µM BaP, 0.25 mM Ni, 0.625 µM Cr(VI), respectively, and p=0.001 for BaP + Ni + Cr(VI), Fisher's Exact Test). However, in this case, the combination of the 3 components caused lower micronuclei frequency than, for example, the Cr(VI) concentration though this difference is not statistically significant (p=0.164), and the mixture also has no significant differences from the other two individual components (BaP and Ni) (p>0.05). The positive control (MMC), with 40 micronucleated cells per 1000 binucleated cells (p<0.001).

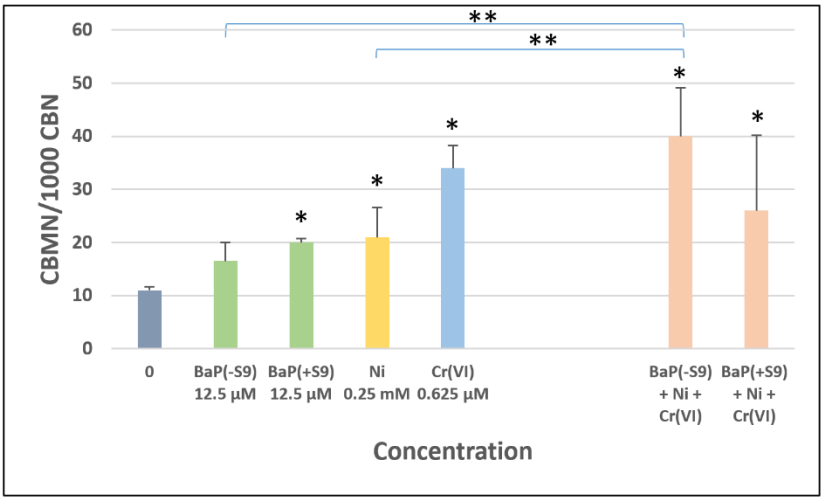


Figure 4.18 - Genotoxicity results (MN assay) in A549 cells exposed to BaP (with and without S9 fraction), Ni, Cr(VI) and a ternary mixture of these compounds, for 24h. Positive control (MMC) had a value of 40 micronucleated cells per 1000 binucleated cells. * indicates significant differences from the negative control. ** indicates significant differences between the mixture and corresponding single substances.

No evident effects of single components and mixture were observed on the CBPI and RI (figure 4.19a, 4.19b).

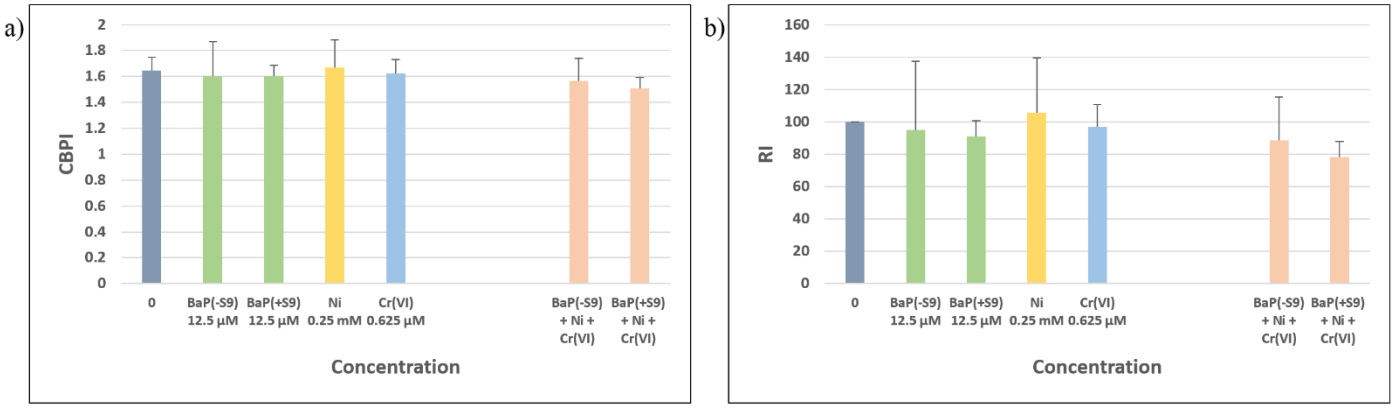


Figure 4.19 - a) CBPI and b) RI results in A549 cells exposed to BaP (with and without S9 fraction), Ni, Cr(VI) and a ternary mixture of these compounds, for 24h.

4.3. Results summary

A summary of the results obtained in the different tests performed is shown in Table 4.1.

Table 4.1 - Cr(VI), Ni and BaP results for MTT and MN assay. CA - concentration addition model; IA - independent action model.

A549 cells	Hexavalent Chromium		Nickel		Benzo(a)Pyrene		Binary mixtures			Ternary mixture	
	24h	48h	24h	48h	24h	48h	24h		48h	24h	48h
MTT assay	Positive cytotoxic results	Positive cytotoxic results	Positive cytotoxic results	Positive cytotoxic results	Equivocal results	Equivocal results	Antagonism (CA)/Synergism at low concentrations (IA)			Antagonism	Antagonism
CBMN assay	Positive genotoxic results	Not applicable	Positive genotoxic results	Not applicable	Positive genotoxic results	Not applicable	Additivity (CA)/Synergism (IA)		Not applicable	Antagonism	Not applicable

According to OECD guidelines, positive results were considered when there was a statistically significant increase in at least one of the tested concentrations, as well as a dose-response relationship. In case of a statistically significant increase in at least one concentration assessed without the existence of a dose-response relationship, the results were considered equivocal.

5. Discussion

5.1. Hexavalent Chromium cytotoxicity and genotoxicity

For the assessment of cytotoxicity and genotoxicity of Cr(VI), the MTT and MN assays were performed, respectively. The results indicate that Cr(VI) is cytotoxic and also genotoxic in A549 cells. In fact, Cr(VI) showed a significant effect in cell viability after 24h exposure, which was more pronounced after 48h exposure. For the genotoxic assessment, three concentrations near or below the IC_{50} were used (0.625, 1.25 and 2.5 µM) and all of them proved to be genotoxic in this cell line, by increasing the frequency of micronuclei. From this assay we also obtained CBPI and RI values, where, once again, there's a tendency of decrease with the increase of concentrations, showing an effect in the cell cycle progression, caused by Cr(VI). For both assays it was possible to observe a concentration-effect relationship and for MTT assay, since it was performed for 24 and 48h exposure, a time-effect response was also observed.

These results go in favor of the majority of authors that have been studying Cr compounds effects both in an occupational context and in *in vitro* models. In a study with small airways epithelial cells, SAEC and BEAS-2B exposed to potassium dichromate in a concentration range of 0.2 µM-200 µM, MTT assay showed a decrease in cell viability starting in 20 µM in SAEC and a dose dependent viability loss starting at the lower concentration of 0.2 µM for BEAS- 2B cells, after 48h of exposure (Pascal *et al*., 2004). BEAS-2B cells were also used in another study with lower concentrations of 1 and 2 µM of Cr(VI). The MTT assay showed, once again, a decrease in cell viability at both concentrations after 24h of exposure (Caglieri *et al*., 2008). With A549 cell line, some studies were also performed. In one, A549 cells were exposed to chromium oxide, for 24h. The MTT assay showed a concentration dependent cytotoxicity, and cell viability was reduced to 77.5%, 71.1% and 62.2% for the tested concentrations of 600, 800 and 1000 µg mL-1, respectively (Senapati *et al*., 2015). In another study with A549 and BEAS-2B cells exposed to lower concentrations of sodium chromate, a significant reduction in cell viability was observed after 24h in the treatment with 10 µM, with cell viability reduced to 43.4 and 53.9% in BEAS-2B and A549, respectively (Cavallo *et al*., 2009). In a study with a human osteoblast cell line, MG-63 exposed to potassium dichromate it was observed a dose-and time-dependent decrease in cell viability with 5 µM of Cr(VI) for 24h. After 48h, cell viability decreased at a higher extent, at all concentrations above 1 µM (Monteiro *et al*., 2019). In another study with peripheral blood mononuclear cells (PBMCs), with the neutral red assay (NR assay), the results showed that the concentrations of 1, 10 and 100 µM of Cr(VI) decreased significantly cell viability after 24 and 48h exposure (Akbar *et al*., 2011). Although it is accepted that Cr(VI) is cyto and genotoxic, there's a wide range of concentrations explored by several authors. Some of them have used relatively high concentrations which is the case of a study in RBL-2H3 cells (Rat Basophilic Leukemia cells), where sodium chromate was used in higher concentrations (50, 100, 200, 300 and 500 µM), for 24h and a decrease in cell viability was observed in all tested concentrations through MTS assay (Eze *et al*., 2019). However, in an occupational and environmental context, lower doses, like the ones used in the present work, are more relevant.

It is important to refer that in the majority of these works, other tests were also performed, including ROS and apoptotic assessment, where both of them also increased along with the tested concentration. Those tests are also very important when explaining and studying the mechanisms behind the cytotoxicity and genotoxicity of Cr(VI) compounds. The cytotoxicity results presented in this work are compatible with a mitochondrial damage provoked by the exposure to Cr(VI), given that the MTT assay measures the ability of living cells to reduce tetrazolium into formazan through mitochondria enzymes, and through this reaction it is possible to determine and evaluate the normal mitochondrial activity. The mitochondrial damage can be justified by the ability of Cr(VI) to readily enter the cells where the detoxification mechanisms start, and Cr(VI) metabolism products are originated (Chen *et al*.,

2019a). These detoxification mechanisms also generate ROS which are considered the principal cause for cellular damages induced by Cr(VI), such as cytotoxicity, DNA lesions and tumor development (Wise *et al*., 2019). At the mitochondrial level, ROS including hydroxyl radicals are known to alter the membrane potential, through the activation of several pathways. This change in the membrane potential is also known to be an early event in apoptosis due to Cr(VI) cytotoxicity (Senapati *et al*., 2015). Another cause for the high levels of cytotoxicity and cell death defended by several studies, due to the ROS formation, is the disruption of the thioredoxin antioxidant system which promotes cell survival by defending against oxidative stress. The accumulation of ROS and the elevated levels of oxidative stress contribute not only to cell cytotoxicity, but also to genetic instability and probable DNA damages (Myers and Myers, 2009).

The results of the micronucleus assay show a high level of genotoxicity and are in agreement with several studies. In a study with A549 and BEAS-2B cells, the comet assay showed a significant induction of DNA damage at 0.5, 1 and 10 µM of sodium chromate after 1 and 4h of exposure (A549 cells), and after 30 minutes at 1 µM, and 1h at 0.5, 1 and 10 µM (BEAS-2B cells). In both cell lines it could be observed an increase in oxidative stress which led to an increase in oxidative DNA damage. According to the authors, this information suggests that the first cell response to Cr(VI) is an increase in the oxidative stress caused by ROS formed by the reduction of the chemical (Cavallo *et al*., 2009). Another interesting work performed in human osteoblast cell line MG-63 exposed to potassium dichromate for 24h showed a significant increase in the micronucleus frequency at lower concentrations of 0.1, 0.5 and 1 µM. The authors also observed a decrease in cell growth probably related to cell cycle arrest. Depending on the cell line and exposure conditions, Cr(VI) compounds are known to be able to alter the cell cycle at different phases (Monteiro *et al*., 2019). In a study with A549 cells exposed for 24h to 1-25 µM Cr(VI), the authors observed cell cycle arrest at the G2 phase, which was associated with the generation of hydroxyl radical and Cr(VI) reductive intermediates, such as Cr(V) and Cr(IV) (Zhang *et al*., 2001). These results support the ones obtained in this work through CBPI and RI analysis as previously referred. There are also several studies with nanoparticles that even though are not completely comparable with the presented results, are still important for Cr toxicity assessment. A549 cells were used in a study where genotoxicity of chromium oxide nanoparticles was measured with both comet and micronucleus assay. Both of the assays showed genotoxic damage in A549 cells, in a range of concentrations between 200-1000 µg mL-1 for the micronucleus assay and 400-1000 µg mL-1 for comet assay. High levels of ROS were also observed, which once again supports the hypothesis of Cr(VI) DNA damage caused, in first instant, by increasing levels of oxidative stress (Senapati *et al*., 2015).

The ROS generation, through detoxification processes contributes to an increase of structural chromosome abnormalities observed through micronucleus assay. The ROS generated are able to interact with proteins, amino acids, or directly with DNA, causing DNA single or double strand breaks; Cr(VI) can form adducts, as well (Chen *et al*., 2019a). These unrepaired breaks in DNA may result in MN formation during anaphase. Besides DNA and chromosome damages, changes in gene expression via epigenetic alterations have been extensively studied. DNA methylation and histone modifications are the main routes for epigenotoxic damage (Chen et al., 2019a; Chen et al., 2019b). Exposure to Cr(VI) compounds has been associated with changes in various histone markers, including the increase levels of H3K9Me2 which have been reported in a study with A549 cells. The increased levels in this histone marker, in turn, will decrease the expression of the mismatch repair gene MLH1, limiting the DNA repair capacity of the cell, resulting in unrepaired adducts and strand breaks (Sun *et al*., 2009), reaffirming the results obtained in this work and previous studies.

5.2. Nickel cytotoxicity and genotoxicity

Nickel exposure proved to be both cytotoxic and genotoxic in A549 cells. Through the MTT assay we could observe that Ni had a significant cytotoxic effect for the two exposure times. Similar to Cr(VI), after 48h of exposure cytotoxicity reached higher levels than in 24h, in the same concentrations, showing a time-and dose-effect response, with an IC_{50} of 0.51 mM for 24h and 0.22 mM for 48h. For the genotoxic assessment two concentrations were selected (0.25 and 0.5 mM), both near or under the IC_{50}. We observed a significant increase in the micronucleus frequency for both concentrations, proving the genotoxic properties of Ni. CBPI and RI values were also assessed, and both decreased with the increase of the tested concentration, showing an effect in cell cycle progression and corroborating the cytotoxicity assessment by the MTT assay. Ni also showed a concentration-effect relationship for its genotoxic potential.

These results agree with several studies principally using *in vitro* models. In one study with A549 and H1975 (human lung adenocarcinoma cells), cells were exposed to nickel chloride. MTT assay showed for both cell lines a dose-response effect with a range of concentrations between 0.25 and 1.5 mM, after 24h of exposure (Chiou *et al*., 2014). Other studies with different cell lines were also performed. Human T-lymphocyte Jurkat cells were exposed, in one study, to Ni^{2+} and cells viability was assessed, after 48h, through flow cytometry viability assays and it was observed a decrease below 50% in cells viability in concentrations above 1 mM (Caicedo *et al*., 2007). Cytotoxicity was also assessed in HK-2 cells (human kidney cells) exposed, for 48h, to 160, 320 and 480 μM of nickel (II) acetate. The MTT assay showed a significantly decrease in cell viability with the increase of tested concentrations. When treated with the higher concentration, viability decreased more than 50% (Wang *et al*., 2012). An osteocyte-like cell line, MLO-Y4, was also used to assess cytotoxic properties of nickel. Cells were exposed to Ni ions for 48h and showed a significant increase in cytotoxicity at 0.10 and 0.50 mM (Kanaji *et al*., 2014). Like it was described for Cr(VI), nickel nanoparticles are very used in different toxicity studies. A549 and BEAS-2B cells exposed to nickel nanoparticles, had a significant decrease in cell viability in the tested concentrations (20-100 μg/mL) after 24h of exposure, through MTT and Alamar blue test (Capasso *et al*., 2014). In another study with A549 and nickel nanoparticles, viability was reduced to 95, 73, 56, 38 and 21% for the concentrations of 1, 2, 5, 10 and 25 μg/mL after 24h of exposure (Ahamed, 2011).

Like it was observed for Cr(VI), nickel may also induced mitochondrial damage leading to high levels of cytotoxicity presented in this work. This can be justified in the first place by oxidative stress caused by ROS generation through detoxification processes and also by glutathione depletion. Being glutathione an important antioxidant, decreasing their intracellular levels leads to an ROS induction (Mohamed *et al*., 2018). Besides, nickel also has the ability to mimic gene expression patterns similar to hypoxia, by inhibiting the HIF-prolyl and asparaginyl hydroxylases. Under hypoxic stress, cell proliferation is attenuated and apoptosis is promoted, justifying the high levels of cytotoxicity in the present work and also in the previous studies reported above (Chen *et al*., 2019b).

Our results of the micronucleus assay showed that Ni is genotoxic at the tested concentrations. Several authors have studied the impact of nickel exposure on DNA damage. A549 cells exposed to $NiCl_2$ for 24h showed a significate increase in DNA damage for all the tested concentrations (100, 250, 500, 750 and 1000 μM), assessed through the comet assay in combination with the Fpg enzyme (Schwerdtle and Hartwig, 2002). Human lymphocytes were also used in one study with nickel chloride. Cells were exposed to concentrations between 1 and 10 mM and it was observed a dose dependent increase of the DNA damage, assessed by the comet assay. Associated with this result, ROS generation was also observed and believed to be the main cause for damaged bases and DNA strand breaks (Chen *et al*., 2003). Human T-lymphocyte Jurkat cells exposed to Ni^{2+} for 48h presented significant results in

DNA fragmentation, assessed through the comet assay, starting in the lowest tested concentration of 0.05 mM (Caicedo *et al.*, 2007). Human lymphocytes were also exposed to nickel oxide in another study where genotoxicity was assessed through comet and micronucleus assays at 12.5, 25 and 50 µg/mL for 6, 12 and 18h of exposure. The results of both assays were coincident, and a significant genotoxic damage was caused by this component at the concentrations of 25 and 50 µg/mL after 18h of exposure. Once again, the authors believed in an active role of oxidative stress in cell death and genotoxicity (Dumala *et al*., 2019). Concerning studies performed with Ni nanoparticles, in one study with Chinese hamster V79 cells, comet assay showed significant DNA damage in concentrations of 62, 125, 250 and 500 µg/mL and micronucleus assay showed a significant increase in micronuclei frequency in the tested concentrations of 250 and 500 µg/mL (Carli *et al*., 2018). In another work with Caco-2 cells, genotoxicity was evaluated using the comet assay with concentrations between 15 and 120 µg/mL. Significant results in DNA fragmentation were obtained between 30 and 120 µg/mL (Abudayyak *et al*., 2020).

It is believed that the genotoxic potential of nickel is proportional to its ability to enter cells and increase the levels of nickel ions, intracellularly (Oller, 2002). Although it is known that nickel doesn't show high affinity to DNA, it can still bind to DNA and some proteins. This direct binding promotes the generation of ROS which makes the nickel a redox active metal capable of inducing oxidative stress. In addition, both soluble and insoluble forms have the capacity to provoke cross-links and DNA strand breaks, which may also contribute to their genotoxicity. However, these particular genotoxic events occur mainly at high cytotoxicity conditions (IARC, 2018), direct interaction of Ni with DNA seems, in fact, to be of a minor relevance and therefore indirect effects have been considered of higher relevance and the principal mechanism underlying Ni genotoxicity and carcinogenicity. Another mechanism associated with indirect causes of nickel genotoxicity is related to its epigenetic effects, especially through DNA methylation (IARC, 2018; Chen *et al*., 2019b). Permanent changes in gene expression are very important in any mechanism of genotoxicity and carcinogenesis. Nickel exposure has the capacity to induce a phenomenon called heterochromatin spreading, which consists in the condensation of heterochromatin and its ability to pull nearby genes into heterochromatic regions and finally, silencing their expression (Chen *et al*., 2019b). It is believed that this process can inhibit DNA repair mechanisms, for example, the nucleotide excision repair (NER) pathway, leading to unrepaired breaks (Schwerdtle *et al*., 2002), that contribute to its clastogenicity that is detectable through the micronucleus assay.

5.3. Benzo(a)pyrene cytotoxicity and genotoxicity

Although BaP exposure proved to be genotoxic in A549, no relevant cytotoxic damage was observed in these cells using 0.1, 1, 10, 12.5, 25, 50, 75 and 100 µM. Cell viability wasn't affected by this compound, even at higher concentrations. We couldn't reach the IC_{50} in both exposure times, with or without S9 activation. Relative cytotoxicity remained between 10-30%, never higher than that value. Although a wide concentration range was used (MTT assay) in order to try to reach the IC_{50}, that was not possible (results shown in the annex). On the other hand, BaP proved to be genotoxic in A549 cells. The micronucleus assay was performed with three different concentrations, and a significant increase in the micronucleus frequency was observed in those tested concentrations. Since an IC_{50} value wasn't reached, we used concentrations previously tested in the literature about this compound. CBPI and RI values were also assessed, and the results corroborate the lack of cytotoxicity of BaP. No alterations at the cell cycle level were observed through these results.

The lack of cytotoxicity obtained in the present work is not supported by several other studies performed with this compound using diverse cell lines. For example, in A549 cells, two recent studies

showed the cytotoxic power of this compound. In one of them cells were exposed to 0.8, 1.6 and 3.2 µM of BaP, for 24h. The MTT assay showed a clear cytotoxic effect, in a dose-dependent manner, with all concentrations causing significant decreases in cell viability, reaching values over 50% of cytotoxicity (Bai *et al*., 2017). In the second study, A549 showed significant decrease in cell viability at 20 and 40 µM of BaP, after 48h of exposure (Azari *et al*., 2019). In one study with HepG2 cells (liver carcinoma cells) exposed for 14h to concentrations between 0.16 and 60 µM of BaP, cellular viability, assessed through the MTT assay, started to decreased significantly at 0.25 µM, and cytotoxicity levels reached values over 50% at the higher concentrations (Tarantini *et al*., 2009). In another work, also with HepG2, the cells were exposed to BaP concentrations between 0.1 and 125 µM for 24h. Cytotoxicity was evaluated through NR uptake assay and the results showed a dose-related decrease in cell survival, with all concentrations causing significant cytotoxicity. Reduction of cell viability to about 50% was achieved at 5 µM (Pinto *et al*., 2014). In another recent study with HT-29 human colon cancer cells, the lactate dehydrogenase kit was used to assess the cytotoxicity of BaP in concentrations between 1 and 25 µM for 48 and 96h. The results for both exposure times showed a significant increase in cytotoxicity provoked by BaP (Myers *et al*., 2020).

As we can observe by the review of the previous reported studies, there's a wide range of concentrations of BaP that can cause cytotoxic damage in different cell types. In the present work, the used concentrations are very similar and sometimes, the exact same concentrations used in other studies, with different outcomes than the one presented in this work.

Some studies with A549 proved BaP to be an accelerator of cell proliferation in long term exposure. This fact might be due to the upregulation of EGFR signaling pathway (Epidermal Growth Factor Receptor). It was reported that BaP exposure, in low doses (1µM) increases the expression and phosphorylation of EGFR responsible for cell growth, survival and proliferation. In this way, BaP was proved and believed to enhance the proliferative activity of A549 by acceleration the expression of the EGFR signaling pathway (Kometani *et al*., 2009). This might be one of the processes underlying the lack of cytotoxicity in results presented in this work. The increase in cell proliferation caused by BaP exposure might be "hiding" the cytotoxicity caused by this compound. We also have to consider that we are working with an *in vitro* system and with tumor cells, sometimes with several passages, and that already entail inherent mutations. New cells modifications and mutations can always occur, and somehow might alter and modify results from what was expected. In this case, it can also be an hypothesis to explain the lack of cytotoxicity in A549 cells. Some cellular modification or mutation could occur, and somehow, conferred to A549 cells tolerance to the BaP toxicity.

The micronucleus assay showed positive results in the increase of micronucleus frequency in every tested concentration of BaP, suggesting its genotoxic relevance. Different authors have studied the impact of polycyclic aromatic hydrocarbons, in particular, BaP, in human health and its genotoxic properties through in vitro assays. The majority of results obtained in previous works go in favor to the ones presented here. A549 cells were used in a particular work where genotoxicity was assessed whit a different type of assay, the γH2AX assay. This assay is based in the rapid phosphorylation of serin-139 of histone 2A, which occurs minutes after a double strand break (DBS) occurs. So, this is a genotoxicity assay based on the detection of DBSs, and it is currently widely used to detect genetic damages induced by toxicants (Garcia-Canton *et al*., 2012). A549 were exposed to 3.15, 6.31 and 12.62 µg/mL, for 24h. The assay was performed and it was observed a significant increase of γH2AX levels in all tested concentrations, in this case, with or without S9 fraction addition (Zhang *et al*., 2018). This assay was also performed in HepG2 and LS-174T cells (colon adenocarcinoma cells), and the results were very similar to the previous ones in concentrations from 10^{-7} M for HepG2 and 10^{-8} M for L5-174T cells (Audeber *et al*., 2012). This assay is believed, like micronucleus assay, to be more sensitive, in genotoxic

assessment, when compared with the comet assay. In another study, V79 cells, were exposed, for 24h, to 3.5, 11, 13 and 15 µg/mL of BaP. The results of the micronucleus assay showed a significant increase in micronucleus frequency for all tested concentrations, in a dose-dependent manner (Whitwell *et al*., 2010). HepG2 cells have been also used in a wide range of experiments concerning BaP exposure. In a work with genotoxicity assessment through comet assay, these cells were exposed to 5, 10, 15 and 20 µM of BaP, for 14h. A significant and linear increase in DNA breaks (measured by the comet assay) was observed and it was believed, by the authors, that those breaks were oxidatively generated, showing an important role of oxidative stress and ROS species caused by BaP exposure (Tarantini *et al*., 2009). HepG2 were also used in one study where both comet and micronucleus assays were performed. Cells were exposed, for 24h, to concentrations between 1 and 25 µM of BaP for comet assay, and between 0.5 and 4 µM for micronucleus assay. Both assays were concordant, and all concentrations induced a significant increase of genotoxic damage (increase in olive tail moment for comet assay, and increase in micronucleus frequency in micronucleus assay). Even though both assays detected genotoxic damage, micronucleus assay was considered a more sensitive test, when compared with the comet assay (Severin *et al*., 2003). An interesting study was performed with four different types of cells, AHH-1 (human B lymphocyte cells), MCL-5 (human lymphoblastoid cells), TK6 (lymphoblastoid cells) and HepG2. All four were exposed to concentrations of Bap between 1 and 70 µM, for 24h and genotoxicity was assessed through the micronucleus assay. MCL-5 presented a significant increase in micronuclei frequency in concentrations higher than 3 µM, as well as HepG2 cells. However, HepG2 were only exposed until 10 µM of BaP due to their high cytotoxic sensitivity. AHH-1 cells showed a significant increase in micronucleus frequency in concentrations from 4 µM on. TK6 cells didn't show any increase in micronucleus frequency when compared with the negative control. Genotoxicity was also assessed for a different exposure time, 4h, between 10 and 70 µM. In this case, MCL-5 and HepG2 both showed a significant increase in micronuclei frequency in concentrations from 40 µM for MCL-5 and 20 µM for HepG2 (Shah *et al*., 2016).

It is believed that the genotoxic capacity of PAHs in general and BaP in particular, depends on the metabolic capacity of the target cells, since these compounds may undergo one or two phases of metabolism and the resultant metabolic intermediates are the ones with both genotoxic and carcinogenic properties (Danuta *et al*., 2017). Although the formation of BaP adducts has been extensively accepted as one of the main known mechanism for BaP genotoxicity, the formation of BaP mediated oxidative DNA damage is also an important contributor to indirect genotoxic damage (Park *et al*., 2008). In the present work, since no alteration at cell cycle level was observed (through CBPI and RI assessment), but a significant genotoxic damage was obtained in different concentrations of BaP, we may hypothesize that genotoxicity was caused mainly through indirect mechanisms, namely oxidative DNA damage. During phase one of BaP metabolism, two important different pathways of BaP activation occur. The first is the formation of dihydrodiol epoxides (B(a)P-7,8-*trans*-dihydrodiol) catalyzed by cytochrome P450 enzymes. The second pathway corresponds to the formation of ortho-quinones (B(a)P-7,8-quinone) via catechols oxidation by dihidrodiol epoxides. This is called aldoketo reductase (AKR) pathway (Danuta et al., 2017; Guengerich, 2008). Redox cycling of quinones can lead to ROS formation and oxidative DNA damage, namely DNA strand-breaks. This genotoxic phenomenon has been reported in A549 exposed to different concentrations of BaP (Park *et al*., 2008; IARC, 2010b). Since DNA strand breaks can be assessed trough the common genotoxicity assays, like micronucleus assay, this can be one of the mechanisms behind the high levels of genotoxicity obtained in the present work.

In this work we tested both cytotoxicity and genotoxicity of BaP with and without S9 activation. For both assays the results were very similar with or without the S9 fraction, which lead us to think about the metabolic capacity of A549 cells. In human lung cells, since they present a limited metabolic capacity, PAHs are metabolized by the cytochrome P450 (CYP) superfamily member CYP1A1

(Schwartz *et al*., 2007). Some studies with A549 have reported an increase of CYP1A1 gene expression when exposed to low concentrations (1 µM) of BaP for different exposure times (24, 48 and 72h) (Kometani *et al*., 2009; Billet *et al*., 2008). It is also known that PAHs can induce CYP1A1 expression in human cells via a p-53 dependent mechanism (Wohak *et al*., 2016). Those studies have also demonstrated a sufficient metabolic capacity to generate genotoxic metabolites of BaP even in skin cells, which might be due to the same cause previous studied in A549 (Danuta *et al*., 2017, Henkler *et al*., 2012).

This information suggests that even though A549 cells may not be quite metabolic competent, the exposure to BaP enhances the gene expression of CYP1A1, suggesting cells ability to metabolize this compound. We hypothesize that this might be an explanation for the absence of differences in cell treatment with and without S9 fraction.

5.4. Binary mixtures cytotoxicity and genotoxicity

It was previously described that individually, both Cr(VI) and Ni cause cytotoxic and genotoxic effects in A549 cells. Several concentrations of Ni and Cr(VI) were combined to evaluate potential interactive effects of binary mixtures on A549 cells viability and micronucleus frequency. The experimental data was then modeled to investigate whether the mixture effects were additive or deviate from additivity towards synergism or antagonism.

The choice of the reference mathematical model is generally based on the MoA of the chemicals in the mixture: if a similar MoA is foreseen then the CA model is applied; if dissimilar MoA are expected, then the IA model is more adequate. While both metals' MoA is complex, it is likely that some interactive effects could occurr in co-exposed A549 cells, possibly mediated by the induction of ROS. This led to the assumption that the CA model could be a reasonable mathematical model to apply. However, since Cr(VI) and Ni also act by mechanisms other than oxidative stress, this assumption could be reductionist. Following these mechanistic-driven considerations, instead of making a theoretical choice, both models were applied to better explore the interactions between Cr(VI) and Ni. Modeling of cytotoxicity results based on the CA model, revealed a predominance of an antagonistic effect between Cr(VI) and Ni, although not very pronounced, concluding that the combination of both metals caused lower cytotoxic effects than expected from the sum of individual effects. When the IA model was applied, a dose-level deviation was observed, with a synergistic effect at lower concentrations, shifting to antagonism at higher mixture concentrations (above the IC_{50}).

Although the actual mechanisms that mediate Cr(VI) and Ni toxicities are complex and not fully understood yet, it is known that both substances can induce oxidative stress and impair DNA repair mechanisms. The Cr(VI) and Ni MoA involves ROS generation, through detoxification mechanisms (Chen *et al*., 2019a; Wise *et al*., 2019; Myers and Myers, 2009; Mohamed *et al*., 2018), membrane potential alteration and consequent apoptosis (Senapati *et al*., 2015; (Chen *et al*., 2019b). At genotoxic level, despite a direct interaction of Cr(VI) or Ni with the DNA molecule, ROS generation and oxidative stress are also of high relevance, since ROS can interact and bind directly with DNA and cause DNA adducts and DNA strand breaks (Chen *et al*., 2019a; Chen *et al*., 2019b). Epigenetic mechanisms of Ni or Cr(VI) exposure can also lead to DNA methylation and gene silencing, inhibiting DNA repair mechanisms (Chen *et al*., 2019b; Schwerdtle *et al*., 2002; Sun *et al*., 2009). Thus, assuming the CA as the most conservative and adequate model to apply, the deviation towards antagonism found for the toxicity of the binary mixture is not easy to explain. In addition, using the IA model that assumes different MoA, a similar effect was detected for high concentrations of both metals. This antagonism may be explained, in part, by inherent limitations of the cytotoxicity assay used, since the reduction of

MTT is dependent on the mitochondria metabolic activity. To clarify this point it might be useful to explore other viability assays independent from mitochondrial normal function, for example, the neutral red assay. Besides this hypothesis, both MoAs of Cr(VI) and Ni are complex and there is a lot that is still being studied, especially at the epigenetic level. It is already known that both substances are capable of causing epigenetic alterations relevant to their genotoxic potential, but little is known about how they interact and how that might influence the regulation of gene expression as single substances and after co-exposure. To clarify this finding more studies need to be performed, with a wider range of concentrations and possibly using other assays that may complement the present findings and allow to go further with mechanistic hypothesis. It is interesting to note that epigenetic studies are also very important and might be one of the keys to understand the mechanisms behind co-exposure to different substances, their interactions and their possible joint effects. This combined effect can be explained by the accumulation of ROS, decrease of cell antioxidant defenses and the inhibition of DNA repair that both metals are able to induce. Therefore, the IA model reveals interactive effects that are more in line with the known MoA of both metals. Since exposure to Cr(VI) and Ni in real-life scenarios, mainly in occupational settings, occurs mostly at low doses, these results point to a greater health hazard than expected from the individual effects.

Modeling of genotoxicity results based on the CA model, revealed a clear pattern of additivity. On the other hand, applying the IA model, a deviation to a synergism was observed, revealing, once again, higher genotoxic effects than expected from the sum of individual effects of Cr(VI) and Ni, pointing to a greater health hazard.

5.5 Ternary mixture cytotoxicity and genotoxicity

The lower single concentrations of Cr(VI), Ni and BaP tested in the present work were selected and combined to evaluate potential interactive effects of heavy metals and PAHs, since this co-exposure is likely to occur in occupational settings that might be an health problem. A549 cells were exposed to the referred ternary mixture and viability and micronuclei frequency were evaluated. Like it was referred before, only one ternary mixture was tested and the data generated was insufficient for modeling.

Concerning the ternary mixture, for both cytotoxicity and genotoxicity, we observed an apparent null or even an antagonistic cytotoxic effect of BaP in A549 cells. The micronucleus frequency in the ternary mixture, without S9 fraction was very similar to the one obtained in the binary mixture of Cr(VI) and Ni, at the same concentrations of the two metals. On the other hand, when S9 fraction was added, the frequency decreased in the ternary mixture when compared with Cr(VI) and Ni binary mixture, at the same concentrations of the two metals. Therefore, BaP exerted an apparent antagonistic effect when added to the metals' mixture. BaP, apart from forming DNA adducts also acts through ROS generation and promotion of oxidative stress leading to oxidative genotoxic damages like DNA adducts and DNA strand-breaks. Thus, it was likely that when in co-exposure with two other substances able to cause similar damages through similar pathways, the effects could be either additive or synergistic, which was not the case. In fact, some studies on combined effects of different heavy metals and PAHs have found that heavy metals inhibit the PAHs toxic effects by modifying the expression of CYP1A1 gene (Korashy *et al*., 2008; Peng *et al*., 2015). PAHs are important up-regulators of CYP1 and its up-regulation can be affected by concomitant exposure to metals. Because CYP1A1-mediated metabolism is a prerequisite for BaP genotoxicity, the reduced activity of CYP1A1 caused by heavy metals may result in a decrease of BaP toxicity (Kaminsky, 2006; Peng *et al*., 2015). Currently there are 4 proposed mechanisms by which heavy metals modulates CYP1A1 expression. The first pathway involves induction of oxidative stress, which in turn will modulate Nrf2 (protein that regulates the expression of antioxidant proteins),

NF-kB (protein complex involved in cellular responses to stress, free radicals, heavy metals, etc), and AP-1 (transcription factor involved in gene expression regulation in response to various stimuli such as stress), that will subsequently alter CYP1A1 expression; the second pathway involves direct interaction between heavy metals and CYP1A1 apoprotein, altering its activity; the third pathway is based on the alteration, by heavy metals, of recruitment of different co-activators required for the CYP1A1 gene expression; the fourth and last pathway assumes that heavy metals might alter the intercellular levels and activities of different AhR (aryl hydrocarbon receptor) associated proteins, such as HSP90 (heat shock protein 90), XAP2 (hepatitis B virus X-associated protein), p32 (mitochondrial protein) and ARNT (aryl hydrocarbon receptor nuclear translocator (Anwar-Mohamed *et al*., 2009). AhR is a transcriptional factor that modulates gene expression and a regulator of enzyme such as cytochrome P450s. Due to this information it is possible that BaP genotoxicity, in the present work, might have been diminished by simultaneous exposure to Cr(VI) and Ni, provoking the inhibition of the CYP1A1 gene and disabling BaP metabolism.

6. Conclusions

This study aimed to evaluate the cytotoxicity and genotoxicity of the binary and ternary mixtures of Cr(VI), Ni and BaP comparatively to their single effects, in a pulmonary cell line, A549 cells, creating an *in vitro* model of occupational exposure.

The results regarding Cr(VI) showed that it provoked time-and concentration-dependent cyto- and genotoxic effects, in A549 cells. The toxic effects caused by Cr(VI) are directly related with its MoA which involves ROS generation through detoxification mechanisms and Cr(VI) intermediate states, inducing high levels of oxidative stress. ROS and also Cr(VI) are able to interact and bind with DNA leading to an increase of structural chromosome abnormalities, and epigenetic effects.

Ni also showed positive results regarding its cyto and genotoxicity with a time-and concentration-dependent effect. Ni MoA mainly relies in its ability to provoke oxidative damage by detoxification processes and glutathione depletion, since this substance shows little affinity with DNA. However Ni has high affinity to bind with nuclear proteins, making the epigenetic effects of great relevance to its toxicity.

BaP, contrary to expectations and previous studies, and contrary to the two substances mentioned above, showed only positive results for genotoxicity, not being confirmed as cytotoxic in this work. However, we have to consider that we work with an *in vitro* system and with tumor cells and that they may have developed some cellular resistance to BaP and aren't easily affected at the cytotoxic level. Since no alteration at cell cycle was observed in the present work we may assume that the genotoxicity of this substance is mainly attributed to indirect mechanisms, namely oxidative DNA damage through AKR pathway. There were also no differences in results with or without the addition of S9 fraction, and previous studies have demonstrated that, in A549 cells, exposure to BaP enhances the expression of CYP1A1 gene, increasing cells capacity to metabolize this compound without the necessity of adding the S9 fraction.

Regarding the mixtures effects, modeling of binary mixtures of Cr(VI) and Ni toxicity, with the CA model, revealed an mild antagonistic effect between Cr(VI) and Ni. On the other hand, when modeling with the IA model, a synergistic deviation in lower concentrations was observed, shifting to antagonism at higher concentrations. Genotoxicity modeling with the CA model revealed a pattern of additivity. When the IA model was applied, a deviation to a synergism was observed. Taken into account the similar MoAs of Cr(VI) and Ni, additivity and the synergistic effect are easily understandable, while the pattern of antagonism observed for cytotoxicity is not fully understood. It might be related with limitations of the assay performed (MTT), or with Cr(VI) and Ni MoAs that are not fully understood.

For the ternary mixture of Cr(VI), Ni and BaP, mathematical modeling was not possible but, an apparent antagonistic effect was observed for both cytotoxicity and genotoxicity. This information agrees with an inhibition of BaP toxicity by the heavy metals, by modifying the expression of CYP1A1 gene that is responsible for BaP metabolism, a hypothesis that has been already advanced by several other authors.

In terms of future prospects, it is necessary to explore further the combination of other concentrations, to increase the power of the mathematical modelling. In addition, other relevant endpoints at genotoxicity level may be explored, for example, epigenetic effects, ROS generation and effects on DNA repair. In vivo studies, despite the crescent attempt to minimize their use, are a very important step in these toxicological studies, since they have the advantage of allowing to assess the influence of the organism as a whole instead of just using cells representative of the target organ in the toxic effects of these substances.

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8. Annexes

Annex A – Cytotoxicity results in A549 exposed to higher concentrations of BaP

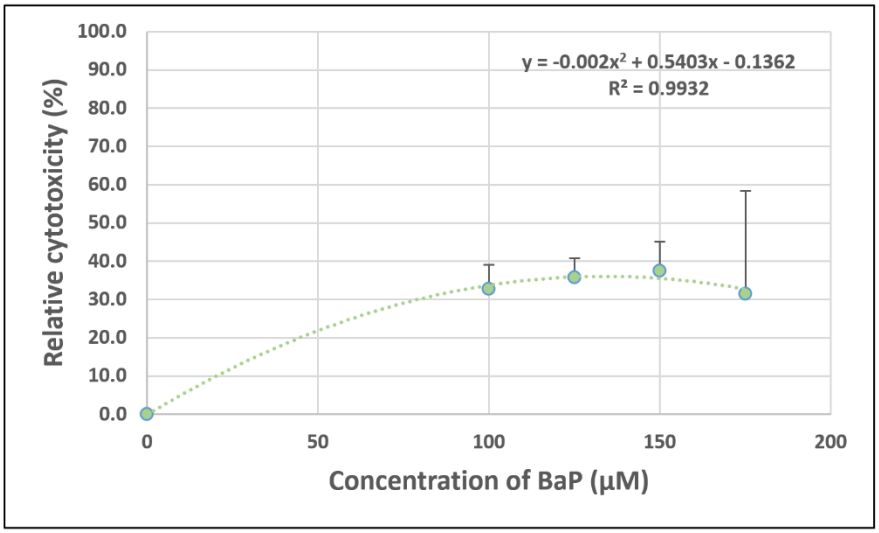

Figure 8.1 - Cytotoxicity results (MTT assay) in A549 cells exposed to BaP.