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Evaluation of Bisphenol A Genotoxicity and Interference on Doxorubicin Effects in HEp-2 and MRC-5 Cell Lines

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List of Acronyms

AURKA	Aurora Kinase A gene
bcl-xl	B-cell lymphoma-extra large gene
BPA	Bisphenol A
c-fos	c-Fos proto-oncogene
CLU	Clusterin gene
DAPI	4′,6-Diamidino-2-phenylindole dihydrochloride
DNA	Deoxyribonucleic Acid
DOX	Doxorubicin
EAS	Endocrine Active Substance
EDC	Endocrine Disrupting Chemical
EFSA	European Food Safety Authority
ER	Estrogen Receptors
ERK/MAPK	Extracellular Regulated Kinase/Mitogen-Activated Protein Kinase
FDA	Food and Drugs Administration
FPG	Formamidopyrimidine DNA glycosylase
GPR30	Trans-membrane Estrogen Receptor
HeLa	Human Cervical Adenocarcinoma Cell Line
HEp-2	Human Epidermoid Carcinoma of the Larynx Cell Lines
IARC	International Agency for Research on Cancer
MN	Micronuclei
MRC-5	Human Lung Foetus Fibroblasts
MT-ND3	Mitochondrially encoded NADH dehydrogenase 3
NER	Nucleotide Excision Repair
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
p21	Cyclin Dependent Kinase Inhibitor 1A gene
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
rpm	Rotations per minute
TDI	Tolerable Daily Intake
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
SCGE	Single Cell Gel Electrophoresis
Std. Dev	Standard Deviation
UV	Ultraviolet
WHO	World Health Organization

Resumo

Introdução: Atualmente vivemos numa sociedade desenvolvida em diversas áreas do conhecimento. Na base deste desenvolvimento, podemos encontrar a indústria química responsável por várias matérias-primas (químicos) que possibilitam a evolução de outras áreas. Este progresso leva a uma diminuição do controlo sobre a libertação destes químicos para o ambiente e, consequentemente torna inevitável permanente exposição de humanos, animais e plantas a estes agentes.
Entre estes químicos encontram-se substâncias que são classificadas como disruptores endócrinos. Esta designação é aplicável a substâncias que apresentam a capacidade de interferir prejudicialmente com o sistema endócrino, através de mecanismos como: simular o comportamento de hormonas, impedir a ligação de hormonas ao respetivo recetor ou diminuir a concentração destas. Estes agentes podem ser encontrados em solventes/lubrificantes industriais, pesticidas, fungicidas, medicamentos, plásticos e plasticizantes. Desta forma, é praticamente inevitável a exposição diária a estes agentes.
O bisfenol A é um dos plasticizantes mais utilizados, podendo ser encontrado em diversos produtos de uso comum como: o papel térmico, a película que reveste a comida enlatada, garrafas de água, entre outros. Este plasticizante é um dos disruptores endócrinos mais estudado. Apresenta uma estrutura semelhante à do estrogénio, sendo assim capaz de ser ligar aos recetores de estrogénio, promovendo ou inibindo a ação desta hormona, dependendo do recetor ao qual estabelece ligação. Este agente químico tem sido associado a diversas alterações como infertilidade, efeitos epigenéticos, cancro, etc.
Cancro é a segunda maior causa de morte no mundo, apresentado um aumento de 42% na mortalidade nos últimos 15 anos. Há vários tratamentos para esta doença como a cirurgia, radioterapia e quimioterapia. Este último baseia na aplicação de agentes antineoplásicos para promover a eliminação das células cancerígenas. A doxorubicina é um dos antineoplásicos mais utilizados, sendo utilizado na terapêutica de vários cancros como leucemias, linfomas, cancro de mama, entre outros.
Tendo em conta a associação do bisfenol A com o desenvolvimento de cancro, levanta-se a questão de saber se este poderá interferir com os efeitos antineoplásicos da doxorubicina e até de outros medicamentos quimioterapêuticos.
Objetivo: Esta investigação pretendeu avaliar os efeitos genotóxicos do bisfenol A, bem como a sua capacidade de interferir com a doxorubicina em linhas celulares humanas de fibroblastos pulmonares de feto (MRC-5) e de carcinoma epidermóide da laringe (HEp-2).
Métodos: Ambas as linhagens celulares foram expostas a baixas doses de bisfenol A (4.4 μM, 4.4 nM, 0.44 nM), a uma dose terapêutica de doxorubicina (4.4 μM) e ainda a ambos os agentes em simultâneo, perfazendo um total de 9 tratamentos [controlo, veículo (exposição ao etanol, solvente do bisfenol A) e 6 exposições]. Recorreu-se à técnica de comet assay para a avaliação do dano no DNA provocado por estas exposições e à modificação desta técnica para avaliar especificamente o dano oxidativo no DNA. Utilizou-se a avaliação citológica de ambas as linhas celulares para determinar o índice mitótico bem como as anomalias mitóticas. Outro método utilizado para avaliação dos danos genómicos foi a avaliação de micronúcleos, permitindo avaliar o dano no DNA que não foi reparado pelos mecanismos de reparação de DNA e persistiu a pelo menos uma divisão celular.
Resultados e Discussão: Avaliando os danos causados pelo bisfenol A *per si* constatou-se que na linha celular HEp-2 a concentração mais baixa apresentou maior dano em comparação com o controlo, estando em acordo com outros estudos que relatam a capacidade deste para causar dano no DNA. O mesmo não foi observado na linha celular MRC-5, que não apresentou diferenças significativas entre as exposições de bisfenol A e o controlo. Este resultado demonstra que o bisfenol A não se comporta da mesma forma em todas as células, o que está em concordância com outros estudos que provam que este agente provoca diferentes respostas dependendo do recetor a que se liga.
No entanto, a linha celular MRC-5 apresentou um aumento significativo do dano oxidativo promovido pela exposição ao bisfenol A, sendo a concentração intermédia (4.4nM) a que mostra maior dano. A

exposição desta linha celular apresenta uma resposta não monotónica, este tipo de resposta é comum no sistema endócrino e já foram anteriormente associadas ao bisfenol A. Podem ser explicadas pela disponibilidade de ligando e de recetor ou seja, se a concentração do ligando é baixa vai levar a que existam recetores livres e assim a resposta não será a máxima; no caso de a concentração ser muito alta não irá existir um aumento de resposta porque todos os recetores estão ocupados, podendo ainda ocorrer que esta concentração seja citotóxica.

Ambas as linhas celulares apresentaram um aumento do dano no DNA decorrente da exposição à doxorubicina, como já era esperado uma vez que o mecanismo de ação deste medicamento baseia-se no aumento do dano para promover a apoptose das células.

Avaliando as exposições a ambos os agentes em simultâneo, verificou-se que ambas as linhas celulares apresentam um decréscimo do dano causado nas co-exposições na concentração mais alta (4.4 μM) e na mais baixa (0.44 nM) de bisfenol A, comparado com a exposição à doxorubicina *per si*. Tendo em conta que os mecanismos de ação da doxorubicina têm como objetivo provocar dano no DNA e evitar que este seja reparado, ao verificarmos que existe uma diminuição do dano causado pelas co-exposições em comparação com o dano provocado pela exposição à doxorubicina *per si*, isto sugere-nos que o bisfenol A tem um efeito antagónico sobre a doxorubicina.

Em relação ao índice mitótico, verificou-se que apenas a linha celular MRC-5 apresenta diferenças significativas em relação ao controlo, demostrando que o bisfenol A tem a capacidade de induzir a divisão celular, como já havia sido descrito por outros autores. O maior índice mitótico foi apresentado pela concentração 4.4 nM. Em ambas as linhas celulares, as células expostas à doxorubicina exclusivamente ou em conjunto com bisfenol A, não apresentaram mitoses. Isto sugere que embora ao nível do dano no DNA o bisfenol A aparenta ter a capacidade de antagonizar os efeitos da doxorubicina, o bisfenol A nestas concentrações não bloqueia o efeito de paragem no ciclo celular promovido por este antineoplásico.

Ambas as linhas celulares apresentam um aumento da percentagem de micronúcleos nas concentrações 4.4 μM e 0.44 nM de bisfenol A, em comparação com o controlo. A exposição à doxorubicina apresentou um decréscimo na percentagem de micronúcleos em relação ao controlo para ambas as linhas celulares. Pode ainda verificar-se em ambas as linhas celulares um aumento, embora não significativo, da percentagem de micronúcleos de algumas das exposições a ambos os agentes em relação ao controlo. Isto demonstra que o bisfenol A apresenta um efeito antagonista sobre a doxorubicina.

Conclusão: Este estudo demonstra que o bisfenol A apresenta efeitos genotóxicos mesmo em baixas concentrações às quais estamos expostos no nosso quotidiano, que diferentes linhas celulares respondem ao bisfenol A de forma diferente e também que este agente provoca respostas não monotónicas.

São ainda apresentadas evidências de que estas concentrações de bisfenol A interferem com os efeitos da doxorubicina a uma concentração terapêutica, podendo ser um fator crucial para indivíduos que estão em tratamento com este agente.

Esta investigação demonstra a relevância de estudar diruptores endócrinos, especialmente os seus efeitos a concentrações baixas que são consideradas como seguras pelas entidades responsáveis pela segurança alimentar (EFSA, Food and Drugs Administration (FDA), etc.) e a importância de encontrar substitutos para estes agentes.

Palavras-chave: Disruptores Endócrinos; Bisfenol A; Genotoxicidade; Agentes Quimioterapêuticos; Doxorubicina.

Abstract

Introduction: The chemical industry has grown in the past few years, leading to the presence of a lot of chemicals in food, air, water and consumer products, thus making daily human exposure to them unavoidable. Some of them are classified as Endocrine Disrupting Chemicals (EDCs). These substances have the ability to change function(s) of the endocrine system by acting on the hormone receptors directly or interfering in proteins that control the delivery of a hormone. Bisphenol A (BPA) is a commonly utilized EDC that has been suggested to interfere with cell division mechanisms.

Objectives: The aim of this *in vitro* study is to evaluate the DNA damage induced by BPA and its interactions with Doxorubicin (DOX) in human lung foetus fibroblasts (MRC-5) and in human epidermoid carcinoma of the larynx cell lines (HEp-2).

Methods: In this study the cells were exposed to low concentrations of BPA (4.4 μM, 4.4 nM, 0.44 nM), to a therapeutic concentration of DOX (4 μM) and to both drugs. After the exposure, genotoxicity was evaluated by comet assay, cytological analysis of mitosis, and micronuclei assay.

Results: Our study shows that BPA induces an increase of DNA damage in the HEp-2 cell line and in the oxidative damage of the MRC-5 cell line. We also found an increase in the mitotic index and the micronuclei percentage in both the cell lines due to BPA exposure. The co-exposures to BPA and DOX of both cell line shows that BPA has a capacity to antagonize DOX effects on DNA damage and micronuclei. The mitotic index was not altered in the co-exposures in comparison to the DOX exposure alone.

Conclusion: This study shows that low-doses of BPA can be genotoxic even without promoting cytotoxicity. Also, it demonstrates that BPA effects are not the same for all cell lines and that BPA interferes with DOX effects at a therapeutic concentration.

Key-words: Endocrine Disruptors Chemicals; Bisphenol A; Genotoxicity; Antineoplastic agents; Doxorubicin.

1. Introduction

This study has its focus on the genotoxic effects of Bisphenol A (BPA) and its interactions with Doxorubicin (DOX).

In the past years, the chemical industry has developed and increased the production of synthetic chemicals, which are used in fertilizers, pesticides, dyes, plastics and others. It is currently impossible to avoid environmental exposure to these chemicals, as they can be found in the air, water, food and consumer products[1–4]. Such perception of the unescapable exposure to these synthetic substances and their known bioaccumulation has led to widespread public concern regarding their possible effects on human life[4].

Endocrine Disruptor Chemicals (EDCs) is a group of synthetic substances that are capable of mimicking hormones (e.g. estrogen) and modulate the endocrine system leading to adverse health effects. Exposure to them can lead to problems of fertility, foetal development, and can also promote effects on certain types of cancer (e.g. breast cancer, ovarian cancer, prostate cancer, thyroid cancer, brain cancer, etc.)[5,6].

BPA is an EDC produced in large scale for the manufacture of polycarbonate plastics and epoxy resins. These plastics and resins have many applications, such as in food and drink packaging, compact discs, bottle tops and water supply pipes and others. It has been reported the involvement of this substance in male infertility, breast cancer, epigenetic effects, etc.[7,8].

Cancer is currently the second leading cause of death worldwide, according to the World Health Organization (WHO) and International Agency for Research on Cancer (IARC), and it was responsible for 6.2 million deaths in 2000 and for 8.8 million deaths in 2015, which represents an increase of 42% in mortality by this disease[9,10]. There are already treatments for cancer such as surgery, radiotherapy, and chemotherapy (e.g. antineoplastic agents)[11]. An example of an antineoplastic agent is DOX, a drug capable of damaging the DNA and promoting the apoptosis of the cancer cells, used for treatment in many types of cancer, such as leukaemia, lymphoma, breast cancer, etc[12].

Nowadays, concern with EDCs such as BPA and the unavoidable environmental exposure to them is increasing. Questions are being raised concerning their effects on humans and if concentrations are a factor in such effects. A better knowledge of their mechanisms of action could help us understand their ability to interfere with the endocrine system and generate epigenetic effects, as well as their possible interference with drugs – like antineoplastic agents – that are administrated to cancer patients with a therapeutic intention. This increasing concern with EDCs and their effects has guided us into our research objectives.

1.1 Objectives

1.1.1 General Objective

The main goal of this investigation was to understand the possible genotoxic effects of low concentrations of BPA in human cells, and evaluate its interference with chemotherapeutic drugs (DOX).

1.1.2 Specific Objectives

To accomplish the general objective, some specific objectives have been established:

- Determine DNA damage and DNA oxidative damage, caused by exposure to BPA, DOX, and a combination of both, in Laryngeal Carcinoma Cells (HEp-2) and Lung Foetus Fibroblasts (MRC-5), by comet assay;

- Determine genotoxicity of exposures to BPA, DOX, and a combination of both, in Laryngeal Carcinoma Cells (HEp-2) and Lung Foetus Fibroblasts (MRC-5), by micronuclei assay;
- Investigate abnormalities in cell division caused by exposure to BPA in Laryngeal Carcinoma Cells (HEp-2) and Lung Foetus Fibroblasts (MRC-5), measuring normal mitosis and abnormal mitosis.

2. State of the Art

Nowadays man-made chemicals are a part of every days' life. Global chemical sales have more than doubled in recent years, rising from about €1 622 billion in 2005 to €3 534 billion in 2015[13,14].
While this industry grows, also does concern with chemical pollution, one of the greatest environmental threats to the planet. Of great concern is the accumulation of industrial chemicals (e.g. mercury, chlordane, benzoperylene) and their potential adverse effects in the food chain and to the endocrine system in humans and wildlife[13,15–17].

2.1 Endocrine Disruptors

The European Food Safety Authority (EFSA) has introduced the term "endocrine active substance (EAS)", to describe any chemical that can interact directly or indirectly with the endocrine system[16]. When these substances result in an adverse effect upon this system or on target organs and tissues, they are acting as EDCs[16].
The EDCs have been defined by WHO (2012): "an endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations"[16]. These substances can interfere with synthesis, secretion, transport, metabolism, binding action or elimination of hormones responsible for homeostasis, reproduction and normal development processes of our body[18,19].
These agents are used in industrial solvents/lubricants (e.g. polychlorinated biphenyls), plastics (e.g. bisphenol A), plasticizers (e.g. phthalates), pesticides (e.g. methoxychlor), fungicides (e.g. vinclozolin) and pharmaceutical agents (e.g. diethylstilbetrol)[8,18,20,21]. Exposure to them turns out to be unavoidable because they are ubiquitous, can be found in food, and water; furthermore, exposition is mostly through contact with the skin, by inhalation, and by mother-offspring transfer (across the placenta or via lactation)[8].
Some important factors that influence the mechanism of action of these agents are: *i)* the window of susceptibility – mammals have some critical periods of development where hormonal control is crucial (like late embryonic, early postnatal and puberty), and the exposure to EDCs during these periods influences the normal development of the individual; *ii)* latency effects – exposure to these substances may not be immediate but rather lead to the development of diseases or disorders later in life; *iii)* mixtures of EDCs – the human being is constantly exposed to mixtures of chemicals and not to a single one in particular, which makes it harder to study their individual effects; *vi)* there are non-traditional dose-response dynamics – in toxicology usually the greater the dosage the more damage is accomplished, but for some substances (like EDCs and hormones) this does not apply, presenting low-dose effects sometimes more powerful than higher ones and some of these chemicals also present a non-monotonic dose-response curve; *v)* transgenerational – these agents are able to not only affect the exposed individual but also its offspring and successive generations, by affecting the germlines and *vi)* epigenetic effects – heritable changes on expression or regulation of genes without any modifications to the DNA sequence[8,18,20–26].
In addition to the daily intake of a diversity of EDCs, it seems that some of them have the ability to accumulate in the lipid compartments of tissues, originating a "body burden" – a term used to refer to the total accumulation of toxins in the body[27,28].
Nowadays, EDCs are known to be involved in the development of a diversity of diseases and disorders such as decrease in fertility, cancer, hormonal disorders, obesity, and diabetes[8,18,20,22,25,29].

2.1.1 Bisphenol A

BPA is one of the most widely applied type of bisphenol, mostly as a monomer in the production of polycarbonate plastics and epoxy resins. It can be found in lots of products of our daily life (e.g. food cans, toys, plastic bottles)[30,31].

BPA was classified as an EDC since the 60's and has been restricted from some products in 2012[6]. This molecule has a structure similar to estrogen, and therefore is capable of mimicking this hormone[32]. BPA binds as an agonist to estrogen receptor β and has agonistic and antagonistic activities at the estrogen receptor α. It also binds to the arylhydrocarbon receptor, and to the thyroid hormone receptor inhibiting its transcriptional activity[31].

BPA is one of the most studied EDC. The U.S. Environmental Protection Reference Dose for Chronic Oral BPA Exposure set the value of Tolerable Daily Intake (TDI) on 50 μg/kg body weight/day[33]. Which was the same set by the European Food Safety Authority until January 2015, but now it has been reduced to 4 μg/kg body weight/day as a temporary TDI until re-evaluation in 2017[34].

BPA presents effects at low-doses – defined as any biological change or damage caused by exposures in a range that humans are typical exposed to, or at lower doses than those tested in traditional toxicology assessments by U.S. National Toxicology Program[23,26,32]. For instance: the exposure to low doses of BPA leads to brain morphologic alterations, interfere with ovarian development and change the expression of genes that control meiosis[6,23,32].

This low-dose effect uncovered that BPA also presents a non-monotonic dose-response curve, meaning that a substance follows a non-linear curve of response, assuming a U-shaped or inverted U-shaped curve[23,26,35]. There are many mechanisms leading to a non-monotonic dose-response curve like: *i)* cytotoxicity – this relays on the observation that hormones can be cytotoxic at high doses and yet change biological endpoints at low doses; *ii)* receptor selectivity – the affinity to a receptor is different for every substance, for instance BPA at low dose binds almost exclusively to the estrogen receptors (ERs), but it can also bind to other hormone receptors although this ligation is weak so it is necessary a higher dose of BPA; *iii)* receptor competition – the EDC compete with natural hormone to the binding site of the receptor[26,35].

These non-monotonic responses occur frequently enough to be ignored, even more if the substances are present in the environment[35].

Exposure to BPA has been described as dangerous, because of its capability to induce in humans and animals some diseases including decreased fertility, early menopause, cardiovascular diseases, diabetes, obesity, abnormal development and cancer[6,35].

2.1.1.1 Reproductive Dysfunction

BPA is able of mimicking estrogen activity, binding to ER and consequently altering the result of the normal hormone binding. These abnormal effects can lead to reproductive dysfunction in females but also in males[24,36].

A study in infertility showed that the number of couples affected by this disorder increased from 42.0 million in 1990 to 48.5 million in 2010[37].

Tian *et al*. (2017) developed a *in vivo* study in mice, demonstrating that BPA exposure leads to injury of the testicules and disruption of spermatogenesis, providing evidence of the toxicity of this EDC on the male reproductive system[38]. It was also reviewed by Williams *et al.* (2014) that exposition to BPA caused testicular changes, including loss of typical lobular structure, reduction of spermatogenic cysts, and lobule diameter in carps; and in mice the exposure to this chemical resulted in slowed meiotic

progression of germ cells as well as decreased quality and quantity of spermatozoa with continuum exposure[39]. This is a less studied subject in humans, and it will be important to understand if there are similar consequences in our species to those observed in animals[40].
The fertility disorders are also found in women. In an epidemiologic study about the association between serum BPA levels and recurrent miscarriage, it was found that mean BPA levels were more than three times higher in women with a history of three or more consecutive first-trimester miscarriages than in women without fertility problems[41]. Ehlich *et al.* (2012) associated high BPA concentrations in urine with reduction in the number of oocytes and normal fertilized oocytes[42].
There are studies demonstrating that exposure to BPA also influence the fetus, since the molecule is capable of crossing the placental barrier. Chou *et al*. (2011) performed a study on healthy pregnant women, and demonstrate that high prenatal BPA exposure increased the risk of lower birth weight and smaller size for gestational age, especially in male infants[43]. It was also reviewed by Roy JR *et al.* (2009) that prenatal exposure to BPA can lead to a precocious puberty in girls and genital abnormalities in boys[44].

2.1.1.2 Epigenetic Effects

Epigenetic modifications are defined as heritable changes on expression or regulation of genes without any modifications to the DNA sequence, but rather a change in chromatin structure and/or the DNA accessibility to transcription factors affecting gene expression[24,25,32,45].
These modifications can be mediated by three major mechanisms: *i)* DNA methylation – is considered a mechanism to lock genes in the "off" position without altering DNA sequence but changing its structure; *ii)* histone modifications – these modifications can be phosphorylation, acetylation, methylation and others, that play a role in the control of gene expression by changing the DNA structure; and *iii)* transcription of non-coding RNAs – non-coding RNAs are involved in gene expression being able to induce or suppress it, they also play an important role in controlling the DNA methylation and histone modifications[24,25,32,46–48].
All these mechanisms have been identified as possible outcomes of exposure to BPA. Weng *et al.* (2010) had contemplated that long-term exposure to BPA can lead to DNA methylation in the promoter regions of lysosomal-associated membrane protein 3 in human primary epithelial cells and breast cancer cells[49]. In another study it was demonstrated that exposure to BPA increase levels of histone methyltransferase Enhancer of Zeste Homolog 2 leading to methylations of histone H3 in human breast cancer cells[45]. Moreover, a study on placental cells revealed that alterations in the expression levels of microRNAs were induced by BPA[50].
These epigenetic alterations can lead to modifications in gene expression, and are prone to cause diseases. It is thus crucial to identify agents that, by promoting epigenetic alterations, jeopardize the normal mechanism of gene expression[47].

2.1.1.3 Cancer

Currently cancer is considered the second leading cause of death worldwide[9]. This disease has many causes and many strategies allow it to evade the cell/organism machinery of cell proliferation control[51]. Though BPA is not classified as a carcinogen, there is plenty of evidence that this chemical is involved in the development of tumors (*e.g.* acting as an aneugen)[52].
The same epigenetic modifications (DNA methylation, histone modifications, non-coding RNAs) associated with BPA exposure have also been associated with changes causing cancer[52]. For example: *in utero* exposure to BPA modifies mammary tissue expression of histone methyltransferase Enhancer

of Zeste Homolog 2 – a histone methyltransferase known to be associated with tumorigenesis. Thus, BPA exposure increases the risk of developing breast cancer[45].
Burks *et al.* (2017) review revealed that exposure to low doses of BPA during fetal development, increases cell proliferation and estrogen sensitivity, reduces apoptosis and modifies the architecture of the mammary gland, predisposing it to carcinogenesis. In addition, other studies have reported that chronic exposure to BPA in various breast cancer cell lines has been able of induce tumor proliferation, epithelial–mesenchymal transition and metastasis[53].
BPA acts as an anti-apoptotic in breast epithelial cells, and it has been described to promote cell survival and proliferation, but that is not its role for all cell types. In pro-myelocytic leukemia and ovarian granulosa cells, this EDC actually induces apoptosis[52]. Studies in male rats established that early life exposure to BPA leads to the development of prostate intraepithelial neoplasia in adulthood, through BPA-dependent epigenetic effects[54]. Chen *et al.* (2014) showed that BPA induces colorectal cancer cells metastasis via induction of epithelial-mesenchymal transitions[55].

2.2 Antineoplastic drugs

An antineoplastic drug (or chemotherapeutic drug) is an agent used in cancer treatment, that can be used alone or in combination with others. These drugs are applied with the objectives of preventing, controlling or stopping neoplasm development[61,62].
The administration of antineoplastic drugs can meet different treatment strategies: *i)* induction – when antineoplastic drugs are the initial therapy with the intent of disease remission; *ii)* consolidation – the agent is administered after disease remission, with the goal of extending freedom from disease and increasing overall survival; *iii)* neo-adjuvant treatment – the drug is used to reduce tumor size before a local treatment (e.g. surgery) is conducted; *iv)* adjuvant treatment – the antineoplastic drug is used in conjugation with another treatment modality such as radiotherapy or surgery; and *v)* salvage therapy or palliative therapy – the antineoplastic agents are used with the purpose of disease control and increase life expectancy[62,63].

2.2.1 Cell Cycle

The cell cycle is the mechanism that allows a cell to divide itself and originate two identical daughter cells[56–58]. This machinery is essential not only for unicellular species allowing their reproduction but also for multicellular species, enabling them to replace the cells that die throughout the organism life. The cell cycle machinery varies among organisms but its main goal is always to pass genetic information to the next generations of cells[56].
In eukaryotic cells, the cell cycle is divided in two phases: Interphase and M (mitotic) phase. The Interphase is by itself divided in three phases: G_1 (first gap phase) – in this phase the cell monitors the extracellular signals and the environment in order to start the synthesis of all the proteins and RNAs needed for DNA replication; S – during this stage the cell duplicates its chromosomes; and G_2 (second gap phase) – during this period the cell verifies the intracellular signals and external environment preparing to enter the M phase. The M phase is divided in 4 phases: prophase, metaphase, anaphase and telophase; and it is at this stage that cell division occurs resulting in the duplication of one cell[56,58,59].

2.2.2 Cell Cycle Checkpoints

A correct cell division is vital for all organisms, it is thus crucial that the cell cycle is properly controlled, in order to avoid or correct errors that would lead to abnormal divisions, production of nonfunctional

organelles, or errors in DNA replication[57,59]. Therefore, the cell cycle presents checkpoints, which are stages at which the cell evaluates internal and external signals to determinate whether the division continues or not[58–60].

The main checkpoints of the cell cycle are G_1 checkpoint, G_2 checkpoint and M checkpoint. At the G_1 checkpoint, the cell checks if internal and external environments like nutrients, molecular signals, and DNA integrity are favorable to the division; if not, it will enter a quiescent state called G_0 phase[56,58–60]. In the G_2 checkpoint, the cell verifies DNA integrity and if its replication was complete; if replication errors or DNA damage is detected, the cell will try to repair these errors and, if damage is irreparable, the cell enters programmed cell death[56,58–60]. The M checkpoint occurs between metaphase and anaphase and at this point the cell verifies if all sister chromatids are correctly attached to the spindle microtubules[56,58–60].

Even when the cell cycle controls are entirely functional, there is a small probability that errors occur in DNA replication. If a DNA replication error or a mutation arises in a key gene for cell division, it can induce uncontrolled cell growth leading to tumor development[58].

2.2.3 Doxorubicin

DOX fits into a class of antineoplastic drugs called the anthracyclines, and is one of the most used drugs for cancer treatment all over the world[64].

The first mechanisms of action proposed for this drug is its intercalation into DNA avoiding the bidding of the topoisomerase-II cleavage, and leading to a DNA double-strand break. Topoisomerase-II is an enzyme that catalyses the unwinding of DNA by cleaving one strand of DNA duplex and passing a second strand of DNA duplex through this transient cleavage[64,65]. The second mechanism of action is based upon doxorubicin oxidation, resulting in doxorubicin semiquinone. This metabolite is unstable and manages to convert back into doxorubicin, a transition which leads to a generation of free radicals that will damage cellular membranes, proteins and DNA[65].

2.3 Genotoxicity

The integrity of DNA is essential for cell fate, since this molecule holds almost all the genetic information in the cell. However, there are several threats to, namely environmental chemicals, UV radiation, errors associated with the machinery of DNA replication, and oxidative damage generated by free radicals released in some reactions of cell metabolism[58,59,66,67].

These factors lead to mutations which may cause diseases, raising the importance of studying the genotoxicity of substances we may get in contact with. The genotoxicity consists in the capacity of a compound to generate damage in DNA[67,68]. It is currently possible to evaluate genotoxicity by resorting to tests for chromosome damage (*e.g.* chromosome aberrations, micronuclei and sister chromatid exchanges) or tests for DNA damage (*e.g.* comet assay, PCR, halo assay, TUNEL)[67,69].

2.3.1 Comet Assay

The comet assay, also known as single cell gel electrophoresis (SCGE), is the most used method for measuring and analyzing DNA damage in eukaryotic cells because of its simplicity, sensitivity and versatility[70,71].

During the 1970s, Peter Cook and his colleagues were pioneers the sedimentation of the nucleoids, which are structures similar to nuclei but depleted of proteins and nuclear membrane[72]. This was achieved by lysing cells in solutions containing non-ionic detergents (which destroy the nuclear and

cellular membranes) and high concentrations of salt (that allows histones and most of the proteins chromatin related to be dissociated from the DNA)[70–72].

The first quantification of DNA damage in single cells embedded in agarose on microscope slides was executed by Rydberg and Johanson in 1978[73]. The procedure consisted in embedding the cells in agarose, on microscope slides, and lysing them under mild alkali conditions, then the cells were neutralized and stained with acridine orange. The DNA damage was measured by the ratio between the fluorescence of double-stranded DNA (presenting green fluorescence) to single-stranded DNA (presenting red fluorescence) detected in a photometer[73,74].

Östling and Johanson benefited from the previous contributions, when in 1984 were the first to present the comet assay as a microelectrophoretic method for direct observation of DNA breakage in single cells. In their technique the cells were embedded in agarose on a microscope slide, lysed by detergents and salts at high concentrations, electrophoresed under neutral conditions, and stained with a fluorescent DNA binding dye[70,71,73–75].

Based on these procedures and the knowledge stemming from the halo assay performed by Roti and Wright in 1987, the method was adapted by Singh *et al.* in 1988. They introduce the alkaline conditions to the lysis and electrophoresis, increasing the sensitivity of the technique to single-stranded DNA breaks[71,74,76].

2.3.1.1 Technique principles

The comet assay is usually used in genotoxicity testing, especially because of its flexibility that allows it to be applied to every type of eukaryotic cell[74].

This assay is based on the high organization and compact structure of undamaged DNA and on the knowing that when DNA suffers damage, its structure destabilizes and organization is lost. The strands of damage DNA lose their compact structure and expand out of the nuclear DNA into the agarose[77].

The method starts by embedding the cells in the agarose to form a gel, then the cells are lysed in a solution containing detergents and high concentrations of salt, which allows to expose nuclear DNA without membranes or nuclear proteins. At this point the gel-embedded cells are no longer cells, these structures are composed by supercoiling DNA and present a similar shape and size to nuclei, they are called nucleoids[70,71,78].

After lyses, the slides are placed in an alkaline solution, pH > 13, allowing the unwinding of the DNA strands. They are then subjected to an electric current, just like in an electrophoresis technique, and the principle is the same. The DNA has a negative charge so in the electric field it migrates towards the anode. Since the undamaged DNA strands are large and compact, they have more difficulty in this migration than the damaged ones. It is thus possible to distinguish the undamaged DNA from the damaged one, because the former will present the DNA compact and intact in a circle, whereas the latter will be extended forming what seems to be a tail (Figure 2.1)[70,71,78].

All steps in this method are relatively simple, but must be conducted with some cautions because the minimal variations can influence the results. The factors that influence DNA migration the most are the concentration of agarose, the duration of the alkaline treatment and the duration as well as the voltage applied in electrophoresis[70,79,80].

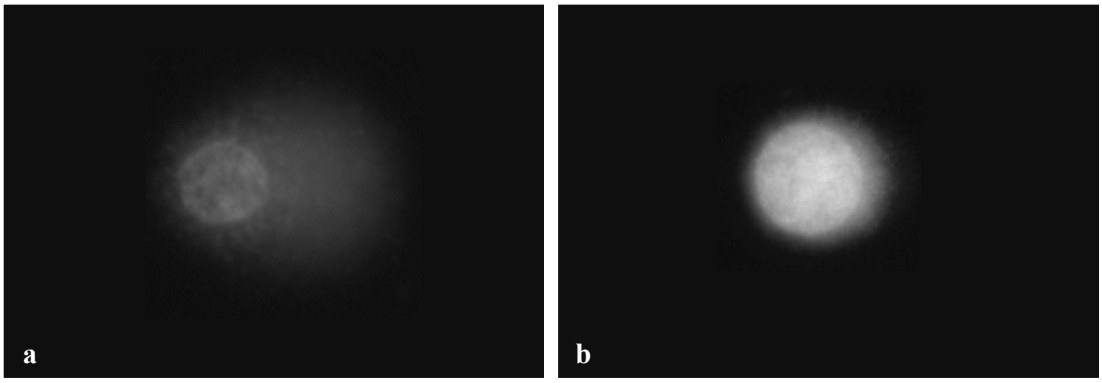

Figure 2.1 - Comets at 400x magnification, stain with DAPI. (a) Comet presenting some DNA damage, (b) cell presenting almost no DNA damage – no comet.

2.3.1.2 Modifications of Comet Assay

Over the years some modifications were introduced to the method so that it could measure oxidative damage, the DNA repair or the antioxidant resistance of cells[70,78].

2.3.1.2.1 Measuring of the Oxidative Damage

To enable the comet assay to measure the oxidative damage of DNA, an additional step was introduced to the protocol. This consists in incubating the slides with an enzyme, just after lysis. This enzyme recognizes specifically oxidized bases and remove them from the DNA chain, creating a break on the strand that will be evaluated in the comet assay. The first enzyme to being used was the Endonuclease III, but nowadays the mostly use one is Formamidopyrimidine DNA glycosylase (FPG). This modification adds other important variables to the protocol which must be taken into consideration – the time of incubation and concentration of the enzyme[70,79,80].

2.3.1.3 Applications

The comet assay can be very versatile, offering the opportunity to be applied in different areas.
Probably the most common application is the genotoxicity testing, a test considered to have high specificity, providing good indication of the genotoxicity of a substance. Therefore the assay is commonly used at the initial screening of pharmaceuticals, cosmetics and other chemicals for possible mutagenic effects[70,74].
The diversity of tissues that comet assay can be applied to, makes it suitable for ecogenotoxicology studies and an increase of its application in this area has been reported [70,74]. Another common application of this assay is in human biomonitoring. Comet assay allows the measurement of DNA damage in populations exposed to a genotoxic agent, or to evaluate the DNA damage due to occupational and environmental exposures[70,80].

2.3.2 *In vitro* Micronuclei Assay

The *in vitro* micronuclei assay is a robust test used to detect micronucleated cells[85]. It can be applied to different cells to measure the chromosomal damage, making this assay very useful in genotoxicity studies[67,85,86].

Micronuclei (MN), also known as Howell-Jolly bodies, were first identified and described in red blood cells by William Howell and Justin Jolly[87]. Later the MN were found to be associated with different pathological states, for example: the formation of MN has been associated with vitamin deficiencies like vitamin B12 and folic acid[87,88].
Exposure to radiation was also associated with the formation of MN. This was first reported in root tip cells and then in lymphocytes[87,88]. These findings led to the classification of MN as a biomarker of DNA damage and this assay can provide intel on the genotoxicity of a chemical or other genomic threats[85,87,89].
MN are defined as fragments or whole chromosomes that separate from the rest of the genome during nuclear division, originating a "micro" aggregate of chromatin detached from the nucleus of the cell[85,86,88–91] (Figure 2.2). The major cause of MN formation is the double-strand DNA breaks, but they can also be caused by failure in chromosomic segregation during the mitotic process[86,91].
MN are originated from acentric chromatid/chromosome fragments or whole chromatids/chromosomes, that during the cell division lag at the anaphase and are not included in the main nucleus[85,87,89].

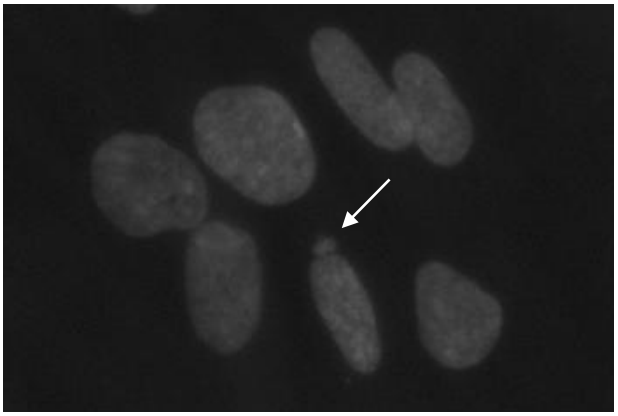

Figure 2.2 - Micronucleus (arrow) at 400x magnification, stain with DAPI.

2.3.2.1 Technique Principle

The *in vitro* MN assay begins with exposure of cell cultures or organisms to the chemical or chemical mixtures under study. During or after this exposure, the cells will have a period of time to allow for cellular growth and chromosome damage or other effect to take place in cell division, which will lead to MN formation. After this period cytokinesis may be inhibited by the actin polymerization inhibitor cytochalasin B resulting in binucleated cells; the evaluation of the MN is made on the cells that have completed one nuclear division. After the period of cell division and inhibition of cytokinesis, cells are harvested, fixed and stained with a nuclear staining, and then evaluated at the microscope by an observer or with an automated screening program[84,85,89].

2.4 Cytological Analysis of Cell Cycle

The cell cycle is crucial to every cell's life, and is also the most complex process which the cell has to go through.
Although it includes strategies (checkpoints) to avoid error, they are not totally error free. For instance, some chemicals are capable of inducing disturbances during mitosis[81,82]. When disturbances in the cell cycle checkpoints occur, they may give rise abnormal chromosome segregation at any mitotic stage;

these abnormalities can be categorized by mitotic polarity/symmetry and segregation of individual sister chromatids[81,83] (Figure 2.3).
The mitotic polarity/symmetry abnormalities include multipolar mitosis and asymmetrical bipolar mitosis. The former are defined by the presence of more than two separate spindle poles. This is strongly associated with supernumerary centrosomes. Asymmetrical bipolar mitosis is an asymmetrical cell division, resulting in chromosomes not being equally divided between the daughter cells[83].
The abnormalities of sister chromatid segregation comprehend *i)* anaphase bridging – this error originates anaphase bridges that are described as a continuous string of chromatin that connects the two poles of the anaphase; *ii)* lagging chromosomes/chromatids – in this case a chromosome/chromatid is left behind at ana/telophase; and *iii)* lagging chromatin fragments – these are DNA fragments without spindle attachment organelles, that are left behind at ana/telophase[83,84].

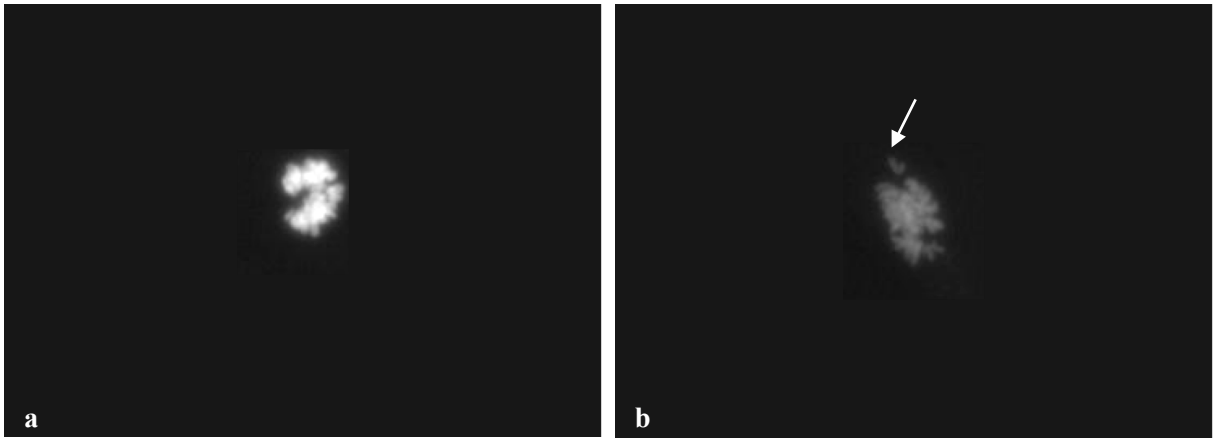

Figure 2.3 - Cell Cycle Abnormalities at 400x magnification, stain with DAPI. (a) Abnormal anaphase; (b) Abnormal Anaphase lagging a chromatin fragment (arrow).

These mitotic errors can cause atypical nuclei in the interphase, that are roughly classified as: *i)* nuclear strings – defined as a chromatin strand connected to the membrane of two nuclei; *ii)* nuclear buds – protrusion of the nuclear membrane linked to the nucleus by a thinner chromatin segment; and *iii)* micronucleus – a chromatin fragment proximal to the nucleus and whose diameter does not exceed one third of the diameter of the nucleus[83].

2.4.1 Analysis of Mitotic Aberrations

Mitotic aberrations are considered important biomarkers of DNA damage, and the analysis of these abnormities is used for determination of genotoxic effects of environmental agents[81].
There are different methods to evaluate these mitotic errors: *i)* chromatin stains – Giemsa, hematoxylin or 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) are dyes usually used to stain the chromatin and can be applied to fixed mitotic cells to allow for microscopic evaluation of mitotic aberrations in monolayer cultured cells; *ii)* chromosome specific staining – this method consists on the application of fluorescence *in situ* hybridisation to allow for visualization of specific chromosomes and score their sister chromatid segregation; *iii)* mitotic proteins staining – immunostaining makes possible to visualise key proteins in the mitotic machinery simultaneously[83].

3. Methods

3.1 Study Design

The design plan of this study is represented in Figure 3.1. The study was conducted with two cell lines, HEp-2 and MRC-5, which were subjected to seven treatments with BPA and DOX, while keeping a set of cells for control. The genotoxic effects of the treatments upon the cells were evaluated by three main techniques – comet assay, MN analysis, cytological analysis of mitosis.
Details on each of these steps are described next.

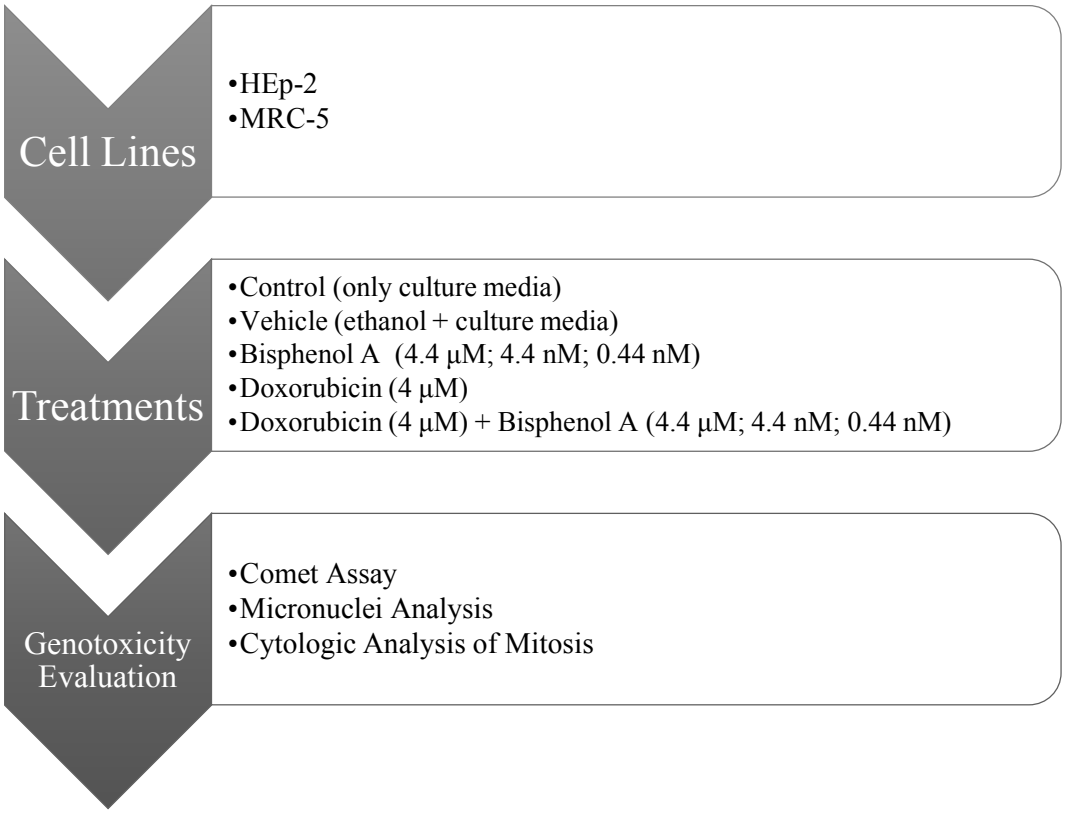

Figure 3.1 - Schematic Representation of the Study Design.

3.1.1 Variables

The impact of three independent variables – concentrations of BPA, a dose of DOX and the mix of BPA and DOX – were evaluated upon a set of dependent variables listed below. This study presents 9 different treatments that consist in the control – no exposure to the drugs; vehicle – exposure to ethanol (the BPA diluent); 3 different concentrations of BPA (4.4 μM, 4.4 nM, 0.44 nM); DOX at a therapeutic dosage of 4 μM; and 3 co-exposures to DOX (4 μM) and BPA (4.4 μM, 4.4 nM, 0.44 nM).
The dependent variables are:

- DNA Damage – measured by the tail intensity of the "comets" in the comet assay;
- Oxidative Damage – measured by the tail intensity of the "comets" in the FPG comet assay;
- Percentage of MN – corresponds to the number of MN counted in 2000 cells;
- Mitotic index – the ratio between the cells undergoing division and the cells not undergoing.

3.2 Cell Lines

HEp-2 has been described as a human epidermoid carcinoma of the larynx cell line. Originally it was thought that they derived from epidermoid carcinoma tissue from the larynx of a 56-year-old male. This cell line is currently known to present HeLa marker chromosomes, which suggests that it has been established via HeLa cell contamination. It presents an epithelial morphology and grows adherent to surfaces[92,93].

MRC-5 is a cell line derived from normal lung tissue of a 14-week-old male foetus. This line presents a fibroblastic morphology and also grows adherent to surfaces[94,95].

3.3 Cell Culture

HEp-2 and MRC-5 cell lines were kindly offered by Centro Hospitalar Lisboa Ocidental - Hospital Egas Moniz, Microbiology and Molecular Biology Laboratory and cultivated in 75 cm^2 flasks with RPMI media containing GlutaMAX™ I, 25 mM HEPES (Invitrogen), supplemented with 10% (v/v) foetal bovine serum, 100 U/mL penicillin, 100 mg/mL streptomycin and 2 mM L-glutamine. Cell cultures were maintained in a 5% (v/v) CO^2 humidified atmosphere at 37°C. After subculture cells were allowed to stabilize for 24 h in a standard growth medium before being subjected to treatments.

3.4 Drugs Exposure

Bisphenol A (Sigma) was freshly diluted in ethanol and added to the culture media to the final concentration of 4.4 μM (1 μg/mL) that corresponds to the tolerable daily intake[34] considering an average body weight of 70 Kg and daily consumption of 3 litres of water; 4.4 nM (1 ng/mL) and 0.44 nM (0.1 ng/mL) correspondent to the range of environmental detected levels of BPA in human biological samples due to environmental exposure[96]. Doxorubicin (AppliChem) was dissolved in water and added to the culture medium to a final concentration of 4 μM (2.5 μg/mL), corresponding to free DOX concentration in blood in clinical cancer chemotherapy[97]. For the combined BPA/DOX exposures cells were pre-exposed to BPA for 24 h followed by an additional 24 h of simultaneous exposure to BPA and DOX. Correspondingly, for single drug exposures cells were incubated with BPA for 48 h after the 24 h stabilization period whereas for DOX standard medium was substituted for medium with DOX 48 h after subculture and maintained. Controls were performed for all experiments using cells grown in standard culture medium or in medium supplemented with ethanol 170 μM (so-called vehicle concentration for BPA).

3.5 Comet Assay

After the treatments, the cells for the comet assay were suspended in a freezing mix (90% v/v of foetal bovine serum and 10% v/v DMSO), frozen at -1ºC/minute in isopropyl alcohol and stored at -80 ºC.

For the analysis of DNA damage and oxidative damage a modification of the comet assay (originally described by Singh *et al.* 1988)[76] was performed to assess the DNA oxidation in this cells as it is described in Collins *et al.* (2012)[98].

The microscope slides were pre-coated with 1% standard agarose (SeaKem®) in phosphate buffered saline (PBS) (Sigma) and allowed to dry at room temperature overnight. The cells were defrosted at 37 ºC and transferred to a 15 mL Falcon tube with 2 mL of PBS (Sigma) and centrifuged at 1500 rpm for 5 mins to eliminate freezing mix. The pellets were diluted in PBS (Sigma) to proceed to cells count using a Neubauer Haemocytometer to a point where the concentration of cells were approximately $2x10^4$ cells /mL.

In a microcentrifuge tube we added 140 µL of 1% low melting-point agarose (LM Pronadisa) in water and 30 µL of cell suspension. Then 70 µL of the mix was taken and transferred as a drop onto the slide (2 drops per slide), each drop was covered with a coverslip (22x22mm) and the slides were left in the fridge for 5 minutes to set the gels. After removing the coverslips, the slides were placed in a lysis solution pH 10 containing 2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris and 1% Triton® X-100, for 1 hour at 4 ºC.

The slides were washed with Buffer F (1 M KCl, 0.5 M EDTA, 0.4 M HEPES and 0.2 mg/L BSA) three times for 5 minutes at 4 ºC, then these slides were incubated with the FPG (kindly donated by Prof. Andrew Collins) for 30 minutes at 37 ºC – these steps were only applied to the slides that we proceed to the oxidative damage detection.

After this treatment, the all slides were submerged with electrophoresis solution – that contains 10 M NaOH and 0.5 M EDTA – for 40 minutes at 4 ºC to the DNA unwinding. Subsequently we performed the electrophoresis in the same solution under 20 V for 20 minutes at 4 ºC. To neutralise the slides, they were washed for 10 minutes with PBS in a coupling jar followed by 10 minutes in water, then the gels were fix with ethanol 70% and ethanol 100% for 5 minutes each, and let dry at room temperature. The slides can be stored for years after this process.

To visualization of the gels, they were stained with 4'6-diamidine-2-phenylindol dihydrochloride (DAPI). The scoring was performed with Comet Assay IV Perceptive Instruments® software, a semi-automated image analysis which is capable of measure the core comet assay measurement parameters like tail length, relative fluorescence intensity of head and tail – which are considered the percentage of DNA in the head and tail, respectively –, etc. In this study will be used the percentage of DNA in the tail since it has been considered the parameter less variable across studies and uses a quantitative measure of damage (from 0 to 100%)[80]. For each gel, 50 cells were scored making a total of 100 per sample. The visualization of the slides was performed by a single observer.

The oxidative damage results from the subtraction of the percentage of DNA in the tail obtain by the alkaline comet assay for that sample from the percentage of DNA in the tail obtain by the comet assay with the FPG treatment, since the enzymatic protocol leads us to the strand breaks and the oxidative breaks[99].

3.6 Micronuclei Analysis

Cells were cultivated on shell vails. After the treatments, cells where fixed with methanol for 20 minutes at room temperature, DAPI stained and mounted on glass slides with mounting medium for evaluation of MN formation. The MN were visualised in a Zeiss Axiovert 40 CFL microscope with immersion oil and 1000x amplification by a single observer according to established criteria for scoring MN: *(i)* the diameter of MN in human lymphocytes usually varies between 1/16th and 1/3rd of the mean diameter of the main nuclei, *(ii)* MN are non-refractile, *(iii)* MN are not connected to the main nucleus, *(iv)* MN may touch but not overlap the nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary, and *(v)* MN usually have the same staining intensity as the main nuclei[84]. For each sample, 2000 cells were scored.

3.7 Cytological Analysis of Mitosis

Cells were cultivated on shell vails. After treatments, cells were fixed in methanol for 20 minutes at room temperature and DAPI stained for evaluation mitotic index of normal and abnormal mitosis.

The cytological analysis of mitosis was visualised in a Zeiss Axiovert 40 CFL microscope with immersion oil and 1000x amplification by a single observer. For each sample, 2000 cells were scored.

3.8 Statistical Analysis

The Kolmogorov-Smirnov test was applied to the comet assay data in order to evaluate the normality of the data. The comet assay results were statistically analyzed with ANOVA test and the ANOVA post hoc test - Games-Howell test. The results of MN Assay and cytological analysis of mitosis were statistically analyzed by Logistic Regression. Student´s t test was used for statistical analysis of all the other parameters.

4. Results

The results of this study are focused in the genotoxic effects of BPA and its interactions with DOX. The results will be presented separately by technique, and within each technique will be divided by cell line in order to facilitate their understanding.
All statistical tests performed in this study used a significance level of 0.05.

4.1 Comet Assay

The results of comet assay are presented by two dependent variables: DNA damage – which corresponds to the damage found in the DNA –, and oxidative damage – a particular type of DNA damage caused by the oxidation of the nucleotides.
These two dependent variables are represented by percentage of DNA in the tail, which is representative of the percentage of damage. The statistical analysis of the comet assay data will compare the means of the percentage of damage resultant of the different treatments.

4.1.1 HEp-2 Cell Line

The DNA damage data in the HEp-2 cell line deviated significantly from normality (Kolmogorov-Smirnov test, $p < 0.05$) for almost all treatments, and the same happened with the oxidative damage data. Nevertheless, graphical examination of the data by histograms suggested that the oxidative damage data are nearly normally distributed, whereas the DNA damage data are not. A square root transformation was then applied to the DNA damage data, leading to results which, although not ideal, still represented a considerable improvement towards data normalization. Variances were not homogeneous either (variance tended to increase with the mean), as indicated by Levene's test ($F(8, 2691) = 13.786$, $p < 0.001$ for DNA damage; $F(8, 2691) = 30.908$, $p < 0.001$ for oxidative damage). A data feature which would later be taken into account when conducting ANOVA.
Average responses to treatments of the two dependent variables, were globally compared by ANOVA and in both cases the null hypothesis of equal mean responses were rejected ($F(8, 2691) = 198.104$, $p < 0.001$ for DNA damage and $F(8, 2691) = 6.898$, $p < 0.001$ for oxidative damage). In spite of this rejection, and because heterogeneity of variance is known to affect the result of ANOVA, the more robust Welch test for equality of means was also conducted. This also rejected the null hypothesis ($F(8, 1120.039) = 226.612$, $p < 0.001$ for DNA damage and $F(8, 1118.913) = 6.736$, $p < 0.001$ for oxidative damage), indicating that there are significant differences among treatments, regarding the mean damages caused to DNA in the HEp-2 cell line. The Table 4.1 shows the means and standard deviations for all treatments.
In order to clarify which treatments caused global rejection of the null hypothesis, repeated comparison of means with the control was conducted by the Games-Howell *post hoc* test, as this nonparametric test does not assume equality of variances and sample sizes[100]. Results of comparisons to the control group for both dependent variables are presented in Figure 4.1.

	Percentage of DNA Damage	Percentage of Oxidative Damage
Treatments	Mean (± Std. Dev)	Mean (±Std. Dev)
Control	15,31 (± 13,81)	9,70 (± 18,66)
Vehicle	11,06 (± 10,32)	3,83 (± 15,61)
BPA 4.4 µM	12,05 (± 11,54)	6,68 (± 15,61)
BPA 4.4 nM	10,14 (± 9,81)	3,63 (±15,61)
BPA 0.44 nM	19,17 (± 14,92)	4,63 (± 15,88)
DOX	37,54 (± 14,85)	5,91 (± 20,72)
DOX + BPA 4.4 µM	31,76 (± 18,32)	9,68 (± 23,20)
DOX + BPA 4.4 nM	33,96 (± 16,81)	12,44 (± 26,18)
DOX + BPA 0.44 nM	38,65 (± 19,34)	7,97 (± 24,26)

Table 4.1 - Mean and Standard Deviation (Std. Dev) of DNA and oxidative damage for HEp-2 cell line.

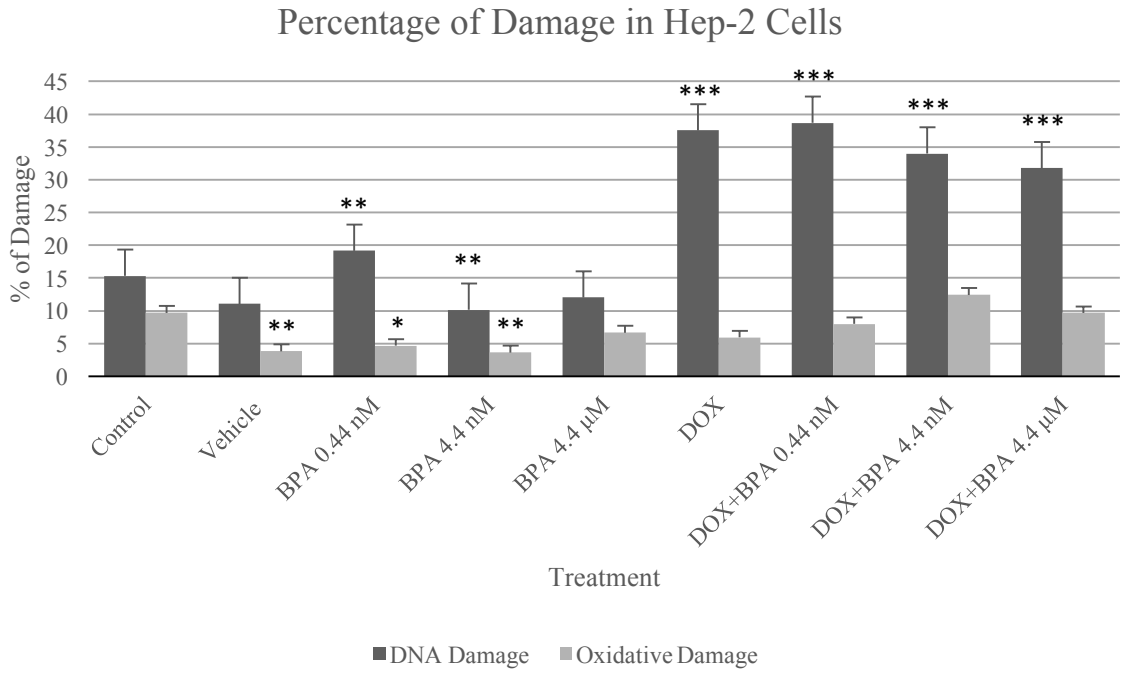

Figure 4.1 - Evaluation of BPA and DOX exposure effects de per se and in co-exposure on DNA damage and oxidative damage in the HEp-2 cell line. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ for ANOVA post hoc test (Games-Howell test) for comparison between treatments and control.

In the Figure 4.1 we can observe the differences between all the treatments and the control. The control group was not exposed to any treatment and the damage found in this group is representative of the damage that occurs in the cell line by any other factors (e.g. UV light, ROS, etc.).

The DNA damage means of DOX and co-exposures of DOX and BPA presented the highest values of damage, being significantly higher than the control, $p < 0.001$. There also a significant increase in the DNA damage presented by BPA 0.44 nM and a significant decrease in the DNA damage presented by BPA 4.4 nM in relation to the control ($p = 0.005$ and $p = 0.001$, respectively).

Relatively to the oxidative damage means, it is perceived that the vehicle, BPA 4.4 nM and BPA 0.44 nM present a significant decrease in comparison to control.
It was also found that the percentages of DNA damage were statistically significant different between the treatment with DOX and the treatment with DOX + BPA 4.4 μM and DOX + BPA 4.4 nM, $p < 0.001$ and $p = 0.036$ respectively.
In the oxidative damage was also found differences statistically significant between the percentages presented by the DOX treatment and the DOX+ BPA 4.4 nM, $p = 0.021$.

4.1.2 MRC-5 Cell Line

The DNA damage data in the MRC-5 cell line also deviated significantly from normality (Kolmogorov-Smirnov test, $p < 0.05$) for almost all treatments. The oxidative damage data however was almost normally distributed for all treatments, to the exception of treatment with BPA 0.44 nM and DOX + BPA 4.4 nM (Kolmogorov-Smirnov test, $p = 0.008$). Graphical examination of the data by histogram, confirmed these results, so the DNA damage data was approximately normalized by a square root transformation. Variances of the DNA damage and oxidative damage data were also found not to be homogeneous (Levene's test: $F(8,891) = 5.399$, $p < 0.001$ for DNA damage and $F(8,891) = 4.159$, $p < 0.001$ for oxidative damage).
The ANOVA revealed that there were statistically significant differences in the means between treatments in both dependent variables ($F(8,891)=13.762$, $p < 0.001$ for DNA damage and $F(8,891) =30.021$, $p < 0.001$ for oxidative damage). Once again, due to heterogeneity of variance, this result was corroborated by the Welch test for equality of means ($F(8, 370.753) = 13.705$, $p < 0.001$ for DNA damage and $F(8,870.761) =28.335$, $p < 0.001$ for oxidative damage) confirming significant differences between treatments. The means and standard deviation are presented in the Table 4.2.

	Percentage of DNA Damage	Percentage ofOxidative Damage
Treatments	Mean (±Std. Dev)	Mean (±Std. Dev)
Control	20,20 (± 13,52)	5,97 (± 18,10)
Vehicle	20,69 (± 15,10)	10,22 (± 19,55)
BPA 4.4 μM	18,62 (± 12,51)	24,95 (± 19,26)
BPA 4.4 nM	19,46 (± 14,26)	31,37 (± 21,18)
BPA 0.44 nM	15,46 (± 9,40)	9,09 (± 16,68)
DOX	25,88 (± 13,32)	10,87 (± 19,56)
DOX + BPA 4.4 μM	11,47 (± 9,70)	1,74 (± 13,85)
DOX + BPA 4.4 nM	22,38 (± 11,14)	3,23 (± 14,53)
DOX + BPA 0.44 nM	17,75 (± 8,60)	14,15 (± 17,89)

Table 4.2 - Mean and Standard Deviation (Std. Dev) of DNA and oxidative damage for MRC-5 cell line.

The Games-Howell *post hoc* test was used for repeated comparisons between treatment means and the control. The results of the comparisons for both dependent variables are presented in Figure 4.2.

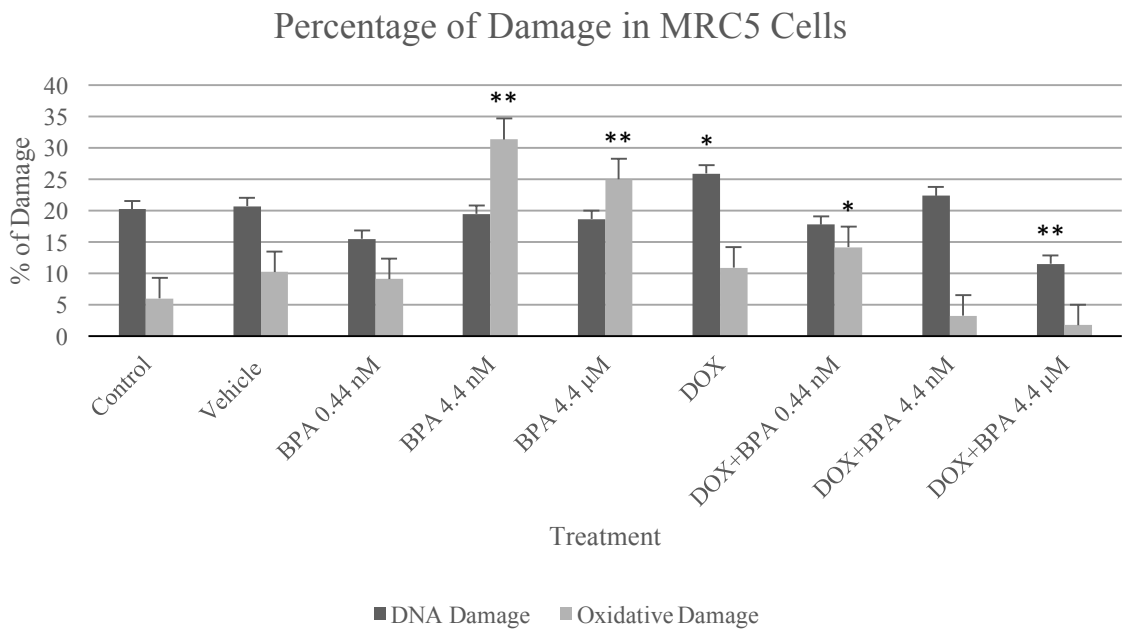

Figure 4.2 - Evaluation of BPA and DOX exposure effects per se and in co-exposure on DNA damage and Oxidative damage on MRC-5 cell line. * $p < 0.05$ and ** $p < 0.001$ for ANOVA post hoc test (Games-Howell test) for comparison between treatments and control.

As in the HEp-2 cells, in this cell line the control group was not exposed to any treatment. The damage found in this group is representative of the damage that occurs in the cell line by any other factors (e.g. UV light, ROS, etc.).
The DNA damage means of DOX presented significantly higher than the control ($p = 0.023$), and is also the highest value of DNA damage. There also a significant decrease in the DNA damage presented by DOX + BPA 4.4 µM in comparison to the control ($p < 0.001$).
Evaluating the oxidative damage, we found that BPA 4.4 µM, BPA 4.4 nM and DOX + BPA 0.44 nM present a significant increase in comparison to control ($p < 0.001$, $p < 0.001$ and $p = 0.04$, respectively).
Comparing the DOX treatment with the co-exposure treatments, we found that the means of DNA damage were statistically significant different between DOX treatment and DOX + BPA 4.4 µM and DOX + BPA 0.44 nM, $p < 0.001$ for both. The percentages of oxidative damage were also statistically significant different when compared the DOX treatment with the DOX + BPA 4.4 µM, $p = 0.006$.

4.2 Micronuclei Assay

The results of MN Assay are express by the percentage of MN observed for each treatment.
The results were statistically analysed by Logistic Regression, using the control group as reference group.

4.2.1 HEp-2 Cell Line

The results for the HEp-2 cell line presented a $p < 0.001$ for the independent variable, this means that the independent variable has influence on the dependent variable. The results of the comparison between the control group and the other groups are presented in Figure 4.3.

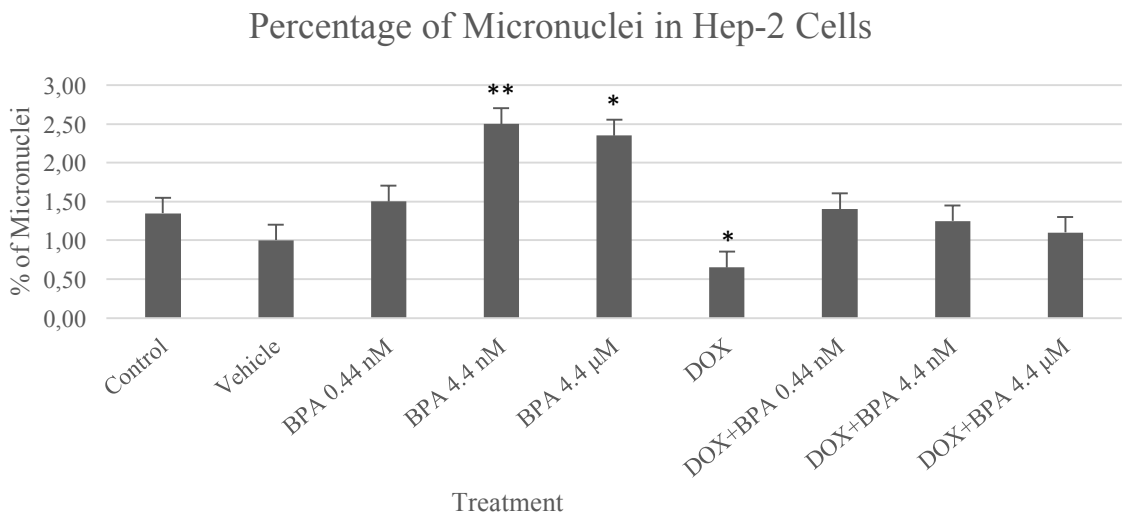

Figure 4.3 - Evaluation of BPA and DOX exposure effects per se and in co-exposure on percentage of Micronuclei on HEp-2 cell line. * $p < 0.05$ and ** $p < 0.01$ for Logistic Regression for comparison between treatments and control.

The BPA 4.4 µM and the BPA 4.4 nM present a significant increase in the percentage of MN in comparison to the control ($p = 0.020$ and $p = 0.009$, respectively). It was also visible in the Figure 4.3, that DOX treatment shown a significant decrease in the percentage of MN in relation to the control.

4.2.2 MRC-5 Cell Line

The results for the MRC-5 cell line presented a $p < 0.001$ for the independent variable, this means that the independent variable has influence on the dependent variable. The results of the comparison between the control group and the other groups are presented in Figure 4.4.

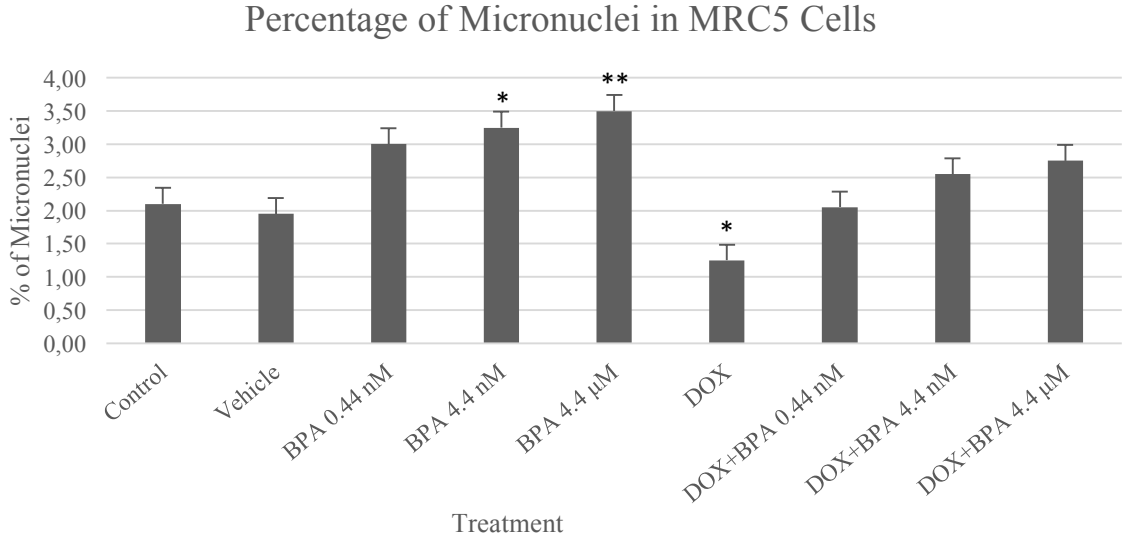

Figure 4.4 - Evaluation of BPA and DOX exposure effects per se and in co-exposure on percentage of Micronuclei on MRC-5 cell line. * $p < 0.05$ and ** $p < 0.01$ and for Logistic Regression for comparison between treatments and control.

Evaluating the comparisons of the treatments with the control, we conclude that BPA 4.4 µM and BPA 4.4 nM shown a significant increase of the percentage of MN ($p = 0.008$ and $p = 0.025$, respectively) and DOX presented a significant decrease in the MN percentage ($p = 0.038$).

4.3 Mitotic Aberrations

The results of mitotic aberrations are expressed by the mitotic index. The mitotic index is the number of cells proceeding with a cellular division divided by the total number of cells in the population in study. This way, our dependent variable would be the mitotic cells.
The results were statistically analysed by Logistic Regression, using the control group as reference group.

4.3.1 HEp-2 Cell Line

The results for the HEp-2 cell line presented a $p = 0.449$ for the independent variable, this means that the independent variable has no influence on the dependent variable. The results of the mitotic index for all the treatments are presented in Figure 4.5.

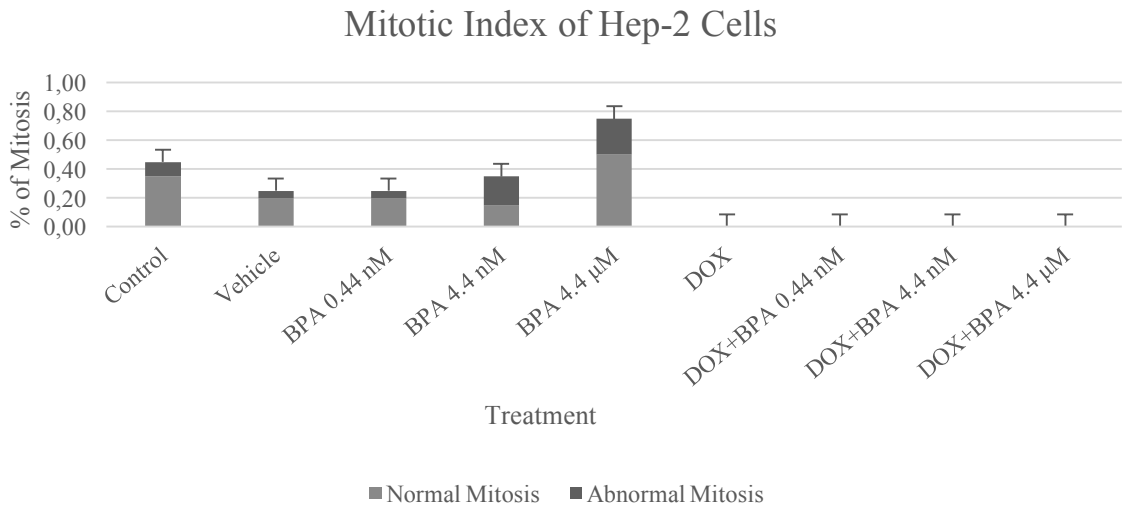

Figure 4.5 - Evaluation of BPA and DOX exposure effects per se and in co-exposure on Mitotic Index on HEp-2 cell line.

This figure show as that BPA 4.4 µM presents the highest percentages of mitotic index, although in comparison to the control there is no significant difference.
There are no significant differences between the control and the other treatments in relation to the abnormal mitotic index. However, the data show that BPA 4.4 µM presents the highest abnormal mitotic percentage.

4.3.2 MRC-5 Cell Line

The results for the MRC-5 cell line presented a $p < 0.001$ for the independent variable, this means that the independent variable has influence on the dependent variable. The results of the comparison between the control group and the other groups are presented in Figure 4.6.

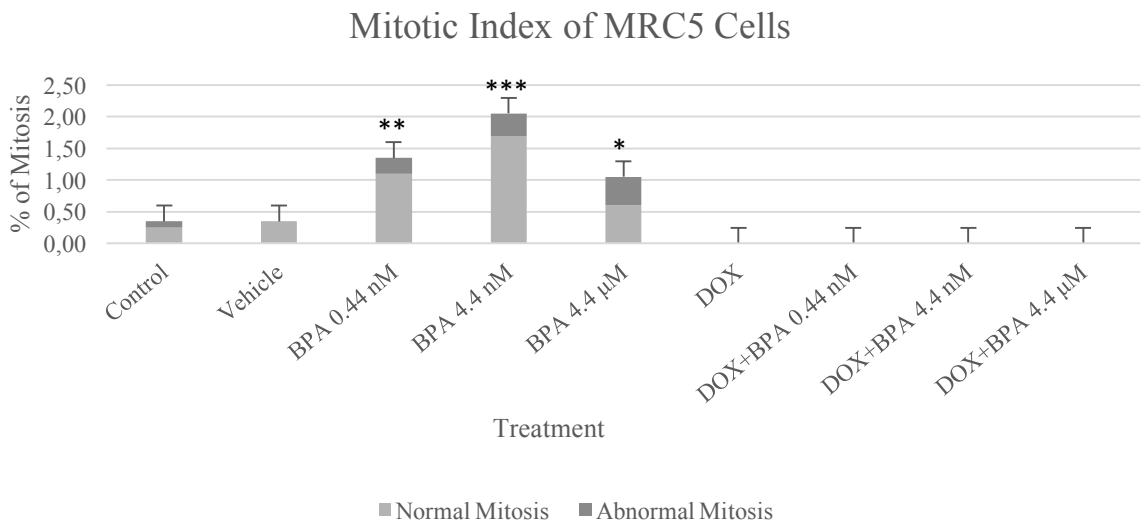

Figure 4.6 - Evaluation of BPA and DOX exposure effects per se and in co-exposure on Mitotic Index on MRC-5 cell line. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ for Logistic Regression for comparison between treatments and control.

As shown in the Figure 4.6, all the BPA exposures present significant increase in comparison to the control. The BPA 4.4 nM has the highest mitotic index.

In relation to the abnormal mitotic index, there were not found significant differences between the treatments and the control. Although BPA 4.4 µM shown the higher abnormal mitotic index.

5. Discussion

This section, discusses the results of BPA genotoxicity and its interactions with DOX. It will be divided by type of genotoxic endpoint: DNA damage, mitotic aberrations, and MN.

5.1 DNA Damage Assess by Comet Assay

The comet assay recognizes DNA damage which is still reparable, such as single and double-strand DNA breaks, alkali labile sites that are converted to strand breaks under alkaline conditions, thus providing information about recent exposure[70,80,101]. Some modifications to the technique also provide information concerning oxidative damage – such modifications resort to an enzyme (e.g. FPG) that converts the nucleotides oxidized into strand breaks[70,80]. It is a method widely used in biomonitoring studies and to evaluate the genotoxic effects of exposure to specific substances (like antineoplastic drugs or chemicals) *in vivo* and *in vitro* studies.

The results of comet assay regarding the exposure of HEp-2 cells to BPA, revealed that the highest DNA damage took place with the lower BPA concentration (0.44 nM), with a mean damage of 19,17 % (±14,92) which is significantly different from the control (15,31%±13,81), $p = 0.005$. These findings are in agreement with other studies demonstrating DNA damage as a consequence of exposure to BPA[102–104]. Iso *et al.* (2006) showed that the capacity of BPA to cause damage in DNA is higher in a cell line expressing ERs, but BPA also causes significant damage in ER-negative cell line[103]. Apparently the binding to ERα mimics estrogenic activity leading to the activation of the extracellular regulated kinase/mitogen-activated protein kinase (ERK/MAPK), whereas the binding to ERß prevents this to signal its downstream targets, acting as an antagonist[105].

It was also observed a significant decrease in DNA damage mean of cells exposed to BPA 4.4 nM (10,14%±9,81) when compared to the control (15,31%±13,81), although it is not significantly lower than those exposed to BPA 4.4 µM. This result may be explained by the non-monotonic response to BPA, as it has been shown and reviewed in some studies[26,106–108]. For instance, Wetherill *et al.* (2002) showed that BPA increased the cell proliferation of prostatic adenocarcinoma cells in a non-monotonic way[108]. These responses are common in the endocrine system, where most non-monotonic responses are explained by hormone concentration and the receptor occupancy[36]. All receptors have a hormone concentration at which their response is maximized, so if hormone concentration is lower than optimal there will be free receptors left; and if it is higher there will be no more receptors to bind so not only will response not increase but also the hormone can become cytotoxic[36].

Viewing the results of cell viability (annexes 1) from this rationale, it appears that even low BPA concentrations that are not cytotoxic can nevertheless lead to genotoxic effects, a finding in concordance with Pfeifer *et al.* (2015)[109]. The concentration of BPA is so low that it does not reach toxic level but it is high enough to bind to the receptor and interfere with cell DNA[36]. Audebert *et al.* (2011) showed that higher concentrations (1-100 µM) of BPA presented cytotoxic effects but not genotoxic ones. This is supportive of the assumption that BPA machinery is similar to hormones, so a high concentration may lead to cytotoxic acute reaction.

In the MRC-5 cell line there was no statistical significant differences between the means of DNA damage in response to BPA treatments and the control. Pfeifer *et al.* (2015) showed that low-doses of BPA up-regulates c-Myc, which induces DNA damage in ERα-negative cells[109]. Dong *et al.* (2011) also described the BPA binding to the trans-membrane estrogen receptor (GPR30) leading to an activation of Erk1/2 in breast cancer cells[110]. This suggests that BPA is not entirely dependent of the ER, which can help explain the absence of significant damage in the MRC-5 cell line – which is an ER-negative cell line[111,112].

Regarding the oxidative damage means presented by the MRC-5 cells after exposure, there are statistically significant differences between BPA 4.4 μM (24,95%±19,26), BPA 4.4 nM (31,37%±21,18) and the control (5,97%±18,10). These findings are in agreement with other studies describing BPA as capable of promoting oxidative damage[113,114]. In the HEp-2 cell line the oxidative damage was significantly lower in the vehicle (3,83%±15,57), BPA 4.4 nM (3,63%±15,61) and BPA 0.44 nM (4,63%±14,92) as compared to the control (9,70%±18,66). These differences were not expected since BPA is known to cause oxidative damage. Since MRC-5 is an ER-negative cell line and HEp-2 is an ER-positive cell line, the possibility arises that these differences are a consequence of the different mechanisms of action of this chemical agent – ER-dependent and independent, as reported by Pfeifer *et al.* (2015)[55,103,109,111,112,115].

The DNA damage of HEp-2 cells caused by exposure to DOX and co-exposure to DOX and BPA was also compared. The mean DNA damage for all DOX treatments was significantly greater than the control mean damage ($p < 0.001$). This can be explained by the DOX mechanisms of action which are based on the induction of damage upon DNA and cause cell cycle arrest[64,65]. These results are in agreement with other studies of exposition to antineoplastic drugs, showing DNA damage caused by DOX[101,116–118].

In the MRC-5 cell line exposure to DOX also led to a significant increase in DNA damage when compared to control. However, the same did not happen in co-exposures to DOX and BPA, as the DOX + BPA 4.4 μM (11,47%±9,70) presented a significantly lower damage than the control (20,20%±13,52). Comparing the exposure of HEp-2 cells to DOX alone with co-exposure to DOX and BPA, the DNA damage of DOX + BPA 4.4 μM (31,76%±18,32) and DOX + BPA 4.4 nM (33,96%±16,81) were significantly lower than DNA damage under DOX alone (37,54%±14,85). Similar decreases were found for the MRC-5 cells, where the DNA damage following exposure to DOX (25,88±13,32) was significantly different from the co-exposures to DOX + BPA 4.4 μM (11,47±9,70) and DOX + BPA 0.44 nM (17,75±8,60) ($p < 0.0001$ for both).

There are studies revealing that BPA antagonizes antineoplastic drugs like DOX, and in this study it was shown that low doses of BPA antagonize the induction of DNA damage by DOX in a therapeutic dose[119,120]. Nevertheless, it does not seem to influence the mitotic index. When we confront the DNA damage of MRC-5, following co-exposure to DOX and BPA with the cell viability assay, it turns out that the co-exposure treatments with less damage are also those which presented a significant increase in cell viability. Saffi *et al.* (2010) showed that the nucleotide excision repair (NER) mechanism is involved in the repair of DNA damage caused by DOX, in this study the MRC-5 cells are defined as NER proficient[121]. This is a possible explanation to the increase in cell viability following exposure to DOX and co-exposures. Although it is noticeable that the DOX + BPA 4.4 nM presented a decrease in viability, this may be due to the antagonist mechanism of DOX by BPA that is still unknown but has already been reported[120].

The means of oxidative damage cause by DOX were not significantly different from control for either cell lines. The oxidative potential of DOX has been previously described[122,123], although Wong *et al.* (2000) showed that the resistance of a cancer cell line to the apoptotic effects of DOX was related with the downregulation of MT-ND3[124]. This raises the possibility that our cell lines also present a mechanism to avoid the oxidative stress cause by DOX.

Nevertheless, the MRC-5 cells presented a significant increase in oxidative damage caused by DOX+ BPA 0.44 nM (14,15±17,88) as compared to control (5,93±18,10). This might be an effect of DOX not yet antagonized by BPA, since the difference was not statistically significant comparing to the oxidative damage cause by DOX (10,87±19,56) – it has been already described that DOX induces oxidative stress[122] –; and at this concentration BPA did not present significant damage.

We also found significant differences between the oxidative damage following DOX exposure (5,91±20,72) and DOX+ BPA 4.4 nM (12,44±26,18) in HEp-2 cells, and the oxidative damage was

significantly lower in DOX + BPA 4.4 µM (1,74±13,85) compared to DOX (10,87±19,56) ($p = 0.006$). This illustrates the interference of BPA in DOX effects[120], and the different effects of BPA depending on cell line as reported by Ribeiro-Varandas (2012)[125] and Aghajanpour-Mir (2016)[126].

5.2 Mitotic Index and Mitotic Evaluation

The statistical analysis of the mitotic index in HEp-2 cells revealed no significant differences between treatments and control.

In the HEp-2 cell line, exposure to BPA 4.4 µM presented the highest percentage of mitosis. It was also visible that cells exposed to BPA 4.4 µM had the highest percentage of abnormal mitoses, but this percentage was not significantly different from the control. This result might perhaps explain why this concentration also exhibited the highest percentage of MN.

The mitotic index of the MRC-5 cell line was influenced by the treatments. Following BPA treatments, the cells presented an increased mitotic index when compared to control, the highest mitotic index being for those exposed to BPA 4.4 nM, $p < 0.0001$. These findings are in concordance with other study which describes an increase mitotic index in response to BPA[127,128].

These studies also described an increase of abnormal mitosis in consequence of exposure to BPA. In our results such increase was visible in both cell lines but not statistically significant[127,128].

Aghajanpour-Mir (2016) showed that BPA causes chromosomal aberrations and their highest values were also in intermediate concentrations of BPA[126].

Both HEp-2 and MRC-5 cell lines did not present mitosis following DOX exposures and co-exposures, suggesting that BPA at these concentrations does not interfere with the cell cycle arrest promoted by DOX[120,129].

5.3 Micronuclei Evaluation

The MN assay allows an assessment of damaged DNA not repaired by the cell, since it is necessary that a cell undergoes at least one division to origin MN.

The MN percentage of the HEp-2 cell line is significantly higher in the BPA 4.4 µM and BPA 4.4 nM as compared to control ($p = 0.02$ and $p = 0.009$, respectively). The analysis of MN percentages in MRC-5 cells revealed an increase of MN formation in the same exposures observed for the HEp-2 cells (BPA 4.4 µM and BPA 4.4 nM), which also presented values significantly different from the control ($p = 0.008$ and $p = 0.025$, respectively). This is in agreement with other researchers that describe BPA as a MN inducer[125,128,130–132].

The formation of MNs following DOX presented a significant decrease comparing to the control in both cell lines, $p < 0.01$.

Interestingly, even though differences were not statistically significant, an increase of MN percentages was observed, in comparison to control, of the DOX + BPA 4.4 µM and DOX + BPA 4.4 nM treatments for the MRC-5 cell line and the DOX + BPA 0.44 nM for the HEp-2 cell line. It was also visible in both cell lines that all co-exposures had a highest percentage of MN than the control. A main conclusion drawing from these results is that BPA antagonizes the DOX effects. This BPA interference upon DOX had already been established by Delgado *et al.* (2015), who described as BPA alters the transcript levels of *AURKA*, *p21*, *CLU*, *c-fos* and *bcl-xl*, all genes related to cell cycle progression, mitotic regulation and apoptosis control[120].

6. Conclusion

In this study it was shown that cell lines do not respond the same way to BPA, contrary to what has been reported in some other studies[125,126].

The cell viability observed in HEp-2 cells seemed related to the DNA damage assessed by comet assay, however the MN percentage was not so concordant. Indeed, the treatments which presented less viability also showed lower MN percentage, and those with highest percentage of MN appears to not influence cell viability.

A non-monotonic response of BPA was observed in the MRC-5 cell line, both in the comet assay and mitotic index results, but this is not found in the MN assay. This may be due to the BPA abnormal mitosis induction, which is greater in the higher concentrations leading to more MN.

It was also concluded, regarding to MRC-5 cell line, that genotoxicity effects are found in non-cytotoxic concentrations.

Our study also provides evidence that low doses of BPA interfere on DOX effects at therapeutic concentrations, which could be a crucial factor for cancer patients undergoing this treatment.

The population is constantly exposed to BPA since it is present in items used on a daily basis like plastic containers, water bottles, thermal printer paper and food cans. This work shows that environmental concentrations of BPA, to which we are all exposed have genotoxic effects. And more importantly, other than genotoxicity, this study also provides evidence for the antagonist effect on DOX – one of the most used antineoplastic drugs in cancer treatment.

7. Future Perspectives

For further investigation, it would be interesting to evaluate the transcript levels of genes like *AURKA*, *p21*, *CLU*, *c-fos* and *bcl-xl*, since their functions are associated with the cell cycle progression or arrest and it has already been shown that their expression is altered when co-exposed to DOX and BPA in a different cell line.

It will be also relevant to evaluate the expression of ER and GPR30, since different mechanisms of action have been reported for BPA depending on these receptors. This could help explain the differences between outcomes of the two cell lines when faced with the same exposures.

Another important study would be biomonitoring cancer patients being treated with DOX, and other antineoplastic agents as monotherapy and in combined therapy, having in mind the evaluation of treatment effectiveness.

8. References

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133. Promega Corporation. CellTiter-Blue ® Cell Viability Assay.

9. Annexes

9.1 Cell Viability

A CellTiter-Blue assay was also performed to assess the cell viability. This assay is based on the metabolic capacity of the cells to reduce resazurin into resorufin which is evaluated by the fluorescence emitted by second one[133].

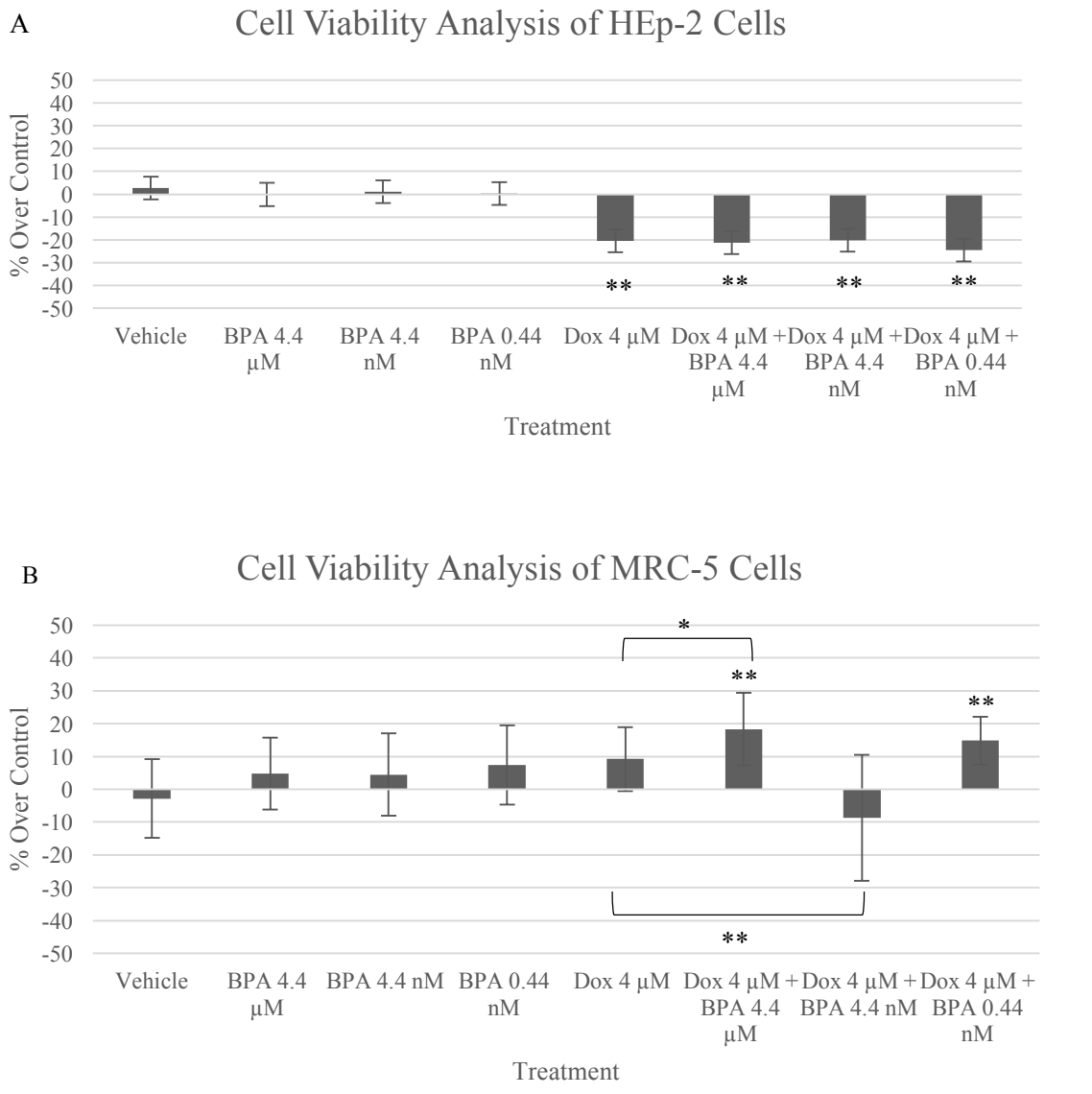

Figure 9.1 - Evaluation of BPA and Doxorubicin exposure effects *per se* and in co-exposure on cellular viability on HEp-2 (A) and MRC-5 (B) cell line. Results are presented as percentage of variation in relation to equivalent cells maintained in standard medium (control). Experiments were repeated on at least three biological replicates, three times per experiment. Student's *t* test ** $p < 0.01$ and * $p < 0.03$ in relation to control and to DOX *per se*.