Universidade de Aveiro Departamento de Biologia
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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, ramo de Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Helena Cristina Correia de Oliveira, Professora Doutora Maria da Conceição Lopes Vieira dos Santos do Departamento de Biologia da Universidade de Aveiro

Dedico este trabalho à Mãe e Irmã e muito especialmente à memória do meu Pai, que infelizmente partiu sem o ver finalizado.
Obrigado por todo o vosso acreditar, pelo vosso apoio, por quem eu verdadeiramente sou**.**

o júri

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agradecimentos

Já antes o disse, somos uma construção de todas as interacções que fazemos durante a vida. No fundo não deixamos de ser uma obra incompleta que nunca será perfeita. Se tiveres um pouco de sorte, um dia encontras alguém que te vê pelo és e que compreende os teus sentimentos. Se fores muito afortunado, encontrarás alguém que te dará a mão e que se sente feliz apenas por te ajudar a subir mais um degrau e a superar mais uma dificuldade. Alguém que fica feliz apenas por partilhar algo de ti.

Ao longo destes anos tive a felicidade de me cruzar com muitas destas pessoas, e é agora chegado o momento de lhes reconhecer a sua amizade e a sua importância no meu percurso.

Às Professoras Doutoras, Maria da Conceição Santos e Helena Oliveira o meu profundo agradecimento por todo o tempo, ajuda e paciência que tiveram e por todo o saber que me transmitiram ao longo destes 2 anos. Sem a vossa ajuda nunca teria conseguido, obrigado por tudo, é para mim um privilégio aprender a/e trabalhar convosco.

A todos os que se cruzaram comigo no laboratório, colegas e todos os outros, o meu obrigado pelas horas bem passadas, pelo companheirismo, pela entreajuda e pelos conselhos e paciência. Um agradecimento especial ao Francisco e Pedro companheiros de percurso à Cristina e à Susana, o meu muito obrigado pela vossa amizade. Um agradecimento muito especial à Andreia, pelos momentos que passamos, pelas trocas de ideias e pelo que aprendi contigo, não tenho palavras para descrever o quanto te devo. Sem todos vós este trabalho não seria o mesmo.

A todos os meus Amigos, agradeço-vos a vossa amizade os momentos de relaxamento, as brincadeiras e todo o nosso convívio. Obrigado Dário por tudo, és um amigo e uma referência para todos nós, à Marau, à Sara à Vera e à Evita, pelos fantásticos anos na Bila e não só. Obrigado César e Diana, à Cátia, à Diana S. e à Sófia, por um primeiro ano fantástico em Aveiro. Obrigado Eva, Joni e Luís pela vossa casa, pela vossa amizade e por todos os momentos de pura descontracção. Obrigado Ana João, Fábio e Vanessa, verdadeiros companheiros de aventura. Este trabalho também é um pouco Vosso.

"Nunca ninguém se perdeu tudo é verdade e caminho."
(Fernando Pessoa)

palavras-chave

Stress oxidativo, MG-63, radicais livres, ROS, superoxido dismutase, catalase,glutatione reductase, apoptose, annexin V, GSH

resumo

O cádmio exerce efeitos nocivos para a saúde pública. A exposição ambiental e ocupacional, são as duas principais formas de exposição em humanos. Foi também demonstrado que mesmo baixos níveis de exposição ao cádmio podem causar falência de diferentes órgãos. A exposição ao cádmio está também associada a doenças relacionadas com o tecido ósseo. Além disso, foi relatado que a exposição ao cádmio pode perturbar a homeostasia do cálcio, formação de vitamina D e é também capaz de interagir directamente no metabolismo ósseo.

As espécies reactivas de oxigénio têm uma função importante na reabsorção óssea e, desta forma, a ruptura do equilíbrio oxidativo pode resultar em distúrbios no seu metabolismo. Embora os efeitos deletérios do cádmio no osso sejam conhecidos, os mecanismos de acção nas células ósseas ainda não estão completamente esclarecidos.

Os objectivos deste estudo consistiram em avaliar o papel do cádmio na promoção de alterações celulares, tais como desequilíbrio oxidativo e indução de apoptose.

Para investigar os efeitos do cádmio foi utilizada a linha humana de osteosarcoma MG-63. A inibição da viabilidade celular pelo cádmio foi avaliada através do ensaio de MTT. Com base nesses resultados foram escolhidos as doses subletais de cádmio (20 e 50 μM) a serem utilizadas nos estudos posteriores. As células foram expostas às duas concentrações de cádmio em dois períodos de tempo (24 e 48 horas). Os seus efeitos citotóxicos foram avaliados vários por parâmetros enzimáticos de stress oxidativo,nomeadamente a determinação da actividade de superóxido dismutase, catalase e a glutationa reductase assim como conteúdo total de glutationa . Além destes, foi também avaliada a indução da apoptose pelo cádmio, através do ensaio com anexina V.

Observaram-se diferenças em todas as enzimas anti-oxidantes, nomeadamente, um aumento da actividade catalase com o aumento da dose de cádmio e uma inibição da actividade da glutationa reductase assim como da concentração de glutationa, resultando em stress oxidativo. Finalmente, observou-se que nas doses e tempos testados, o cádmio induziu apoptose nas células.

Em conclusão, o cádmio, nas concentrações de 20 μM e 50 μM induziu alterações na fisiologia da linha celular humana MG-63, causando stress oxidativo levando à morte celular por apoptose.

keywords

Oxidative stress, MG-63,free radical, ROS, superoxide dismutase, catalase,glutathione reductase, GSH, apoptosis, annexin V

abstract

Cadmium exerts harmful effect on public health. Environmental and occupational exposure, are the two main ways for human exposure. It was also shown that even low levels of cadmium exposure can leave to renal and other organs failure. Exposure to cadmium is associated with various bone problems and diseases like bone loss, fragilization and osteoporosis. Moreover, it was reported that cadmium exposure may perturb calcium homeostasis, Vitamin D formation and interact directly on bone metabolism.

Reactive oxygen species have an important function in bone resorption, so disruption of the oxidative balance in bone tissue may result in disorders in bone metabolism. Although the deleterious effects of cadmium in bone are known, the mechanisms of action in bone cell are still not completely understood.

The aims of this research study were to evaluate cadmium role in promoting cellular alterations, namely inducing oxidative stress imbalance and ultimately apoptosis.

To investigate cadmium effects the human osteoblast-like cell line MG-63 was used. Inhibition of cell viability by cadmium was assessed by MTT assay. Based on these results, the sublethal doses of cadmium, 20 and 50 µM were chosen, for further studies. Cells were exposed to those concentrations of cadmium chloride for two periods of time (24 and 48 hours). After that, cells were harvested and several parameters were evaluated. The cytotoxic parameters followed a comprehensive set of oxidative stress assays with enzymatic parameters, namely determination of the activity of superoxide dismutase, catalase glutathione reductase and total glutathione content. Moreover, cadmium-induced apoptosis was assessed with annexin V assay.

We observed differences in all anti-oxidant enzymes, namely an increase, catalase activities with the increased of cadmium dose and a glutathione reductase activity inhibition and also depletion on glutathione content, which may result in imbalance in oxidative stress. Finally, for the doses and periods tested, cadmium induced cell apoptosis..

In conclusion, cadmium chloride at 20 µM and 50 µM induced changes on human MG-63 cell line physiology, as it cause oxidative stress imbalance which lead to cell death by apoptosis.

Introduction

1. CADMIUM: GENERAL CONSIDERATIONS

Cadmium (Cd) was discovered in XIX century in 1817 by the German chemist Friedrich Strohmeyer. It is a natural element in the earth's crust and usually found in combination with other elements such as oxygen (cadmium oxide), chlorine (cadmium chloride), or sulfur (cadmium sulfate, cadmium sulfide). All soils and rocks, including coal and mineral fertilizers, contain some Cd. Most Cd used is extracted during the production of other metals like zinc, lead and copper (1).

Cd does not corrode easily and has many applications, including the manufacture of nickel-cadmium (Ni-Cd) batteries, pigments and plastic stabilizers and also in metal coatings, alloys and electroplating. It also has applications in nanotechnology, e.g., in cadmium-containing nanoparticles (such as those of CdSe or CdTe), which have numerous biomedical applications, especially in the diagnosis of cancer (2-4). Natural sources of Cd exposure include volcanic activity, forest fires and soil particles carried by wind. Most important anthropic sources are copper (Cu) and nickel (Ni) smelting and fossil fuel combustion (5).

Cd is nowadays classified as a toxic element without any beneficial role in human physiology. It was recently reported that some marine algae contain a form of the enzyme carboanhydrase with Cd instead of zinc (Zn) in their active sites (6).

1.1. Cadmium as a Health Hazard

Cd is an ubiquitous environmental and occupational pollutant. Its first harmful effects were discovered only forty years later of its discover, as acute gastrointestinal and respiratory problems in persons using cadmium carbonate powder as a polishing agent. Soon after 1912, its occupational exposure was augmented with production of Ni-Cd batteries in Sweden. Damage in the lungs in Cd-exposed workers was reported early as the 1930s, later, in the 1940s proteinuria and emphysema was reported and confirmed by further investigations on lungs, kidneys, and bones (recently reviewed by Nordberg (7)). It was based on *Itai-itai* disease that the dangerous dimensions of Cd as an environmental pollutant were demonstrated. *Itai-itai* was an endemic bone disease characterized by fractures and severe pain related to Cd exposure that occurred after World War II in Toyama Prefecture in Japan. By 1968, the Japanese government acknowledged this as an environmental disease related to Cd-contaminated water released from a mine into the Jinzu River, which was used to irrigate rice fields (7). For the first time, Cd pollution was shown to have severe consequences on human health with the demonstration that exposure to high Cd levels was associated with adverse effects on the skeleton through toxic effects on kidney, and also, as was confirmed later, by direct Cd effects on bone tissue. For all that, World Health Organization considers Cd as a highly toxic and a human carcinogen causing cancers in lung, prostate, pancreas, and kidney (8).

1.1.1. Cadmium intake

Humans are prone to make contact with Cd either by occupational and non-ocupational exposure. The routes of its intake involve the lungs, intestines and skin. Cd binds predominantly to metallothioneins (9-11). The Cd–metallothionein complex is distributed to various tissues and organs and is ultimately reabsorbed in kidney tubuli (10, 12). There are no known mechanisms for the excretion of Cd in humans, thus it accumulates in tissues. The half-life of Cd in kidney cortex is 20–35 years. In humans, the largest amount of Cd is deposited in the kidneys, liver, pancreas and lungs (13).

1.1.1.1. Occupational exposure

Occupational exposure to Cd usually occurs in: mines; production of batteries and pigments containing Cd; mineral smelting, Cd production and processing; recycling electronic waste and in smoking. In the last few decades, the production and use of Cd had a significant drop, especially in the United States of America and in the European Union (14). Cd nanoparticles, despite their potential to revolutionize medical therapy, are putative toxicants, and their use is therefore likely to become another source of its toxicity in the future. However, Cd remains a major health problem, mostly due to its long half-life (15 to 20 years) and persistence in the environment. It can enter the food chain, increasing Cd human availability by bioaccumulation (15).

1.1.1.2. Non- occupational exposure

Food is the main source of Cd for the non-smoking population estimates of dietary Cd intake worldwide range from 10–40 μg/day in non-polluted areas to several hundred micrograms in Cd-polluted regions (16). Cd is a contaminant present in almost all forms of food, with greater incidence to wheat and rice, green leafy vegetables, in potatoes and in fish and jellyfish (mollusks and crustaceans). This is due to anthropic activities (as those reported above) which contribute in a significantly manner to Cd leakage to food chain by the soil and seas contamination. Additionally, the predominant human Cd daily intake arrives from smoking. Depending of the brand, each cigarette can contain between 1 μg and 2 μg of Cd, which 40% to 60% can reach the blood stream by oral intake (17, 18).

1.1.2. Cadmium and bone

As reported above, the consumption of Cd-contaminated rice and water provoked severe problems in Toyama-Japan population. *Itai-itai* etiology consists in symptoms of osteomalacia and osteoporosis, femoral pain, lumbago and skeleton deformations, renal tubular dysfunction, immune deficiencies and anaemia. Biochemical findings were characteristic of osteomalacia with increased serum levels of alkaline phosphatase and decreases in calcium and phosphate levels. Anemia and gastrointestinal and renal dysfunction were other less prominent findings (7)

To define the occupational and environmental exposure risks, the Scientific Committee on the Toxicology of Metals under the International Commission on Occupational Health established some key concepts. One of them is the term, "critical organ" which is considered as "that particular organ which first attains the critical concentration of a metal under specified circumstances of exposure and for a given population". The "critical organ" is related to the term "critical effect". The "critical effect" is the earliest adverse effect occurring at relatively low exposures when the threshold concentration (critical concentration) is reached in the critical organ. The critical concentration in the critical organ is the concentration in the organ when adverse functional changes, reversible or irreversible, occur in the most sensitive cells of the organ (as described by Nordberg (7)). The knowledge of the critical effect is crucial for preventive action, because it is the earliest effect occurring at the lowest exposure levels (7).

During the first half of the last century, damages of lung and kidney were reported as the critical effects in occupational exposure to Cd. Researchers initially considered bone effects as secondary ones (mediated by kidney effects). The Cd effects on bone thus were not considered to be critical effects. At present, it seems more difficult to determine whether bone effects may be critical effects or not, because there are indications from animal experiments of a direct effect of Cd on bone remodeling, and increased bone loss has been demonstrated in Cd exposed animals even before renal dysfunction is developed. These data support therefore that Cd has a direct effect on bone mineralization (Bhattacharyya *vide* Gunnar (19)).

In the last decade emerging new data arose on Cd toxicity on several tissues/organs such as leading to impairment of reproductive system (20) and promoting carcinogenesis (see 1.2), among others. Similarly, direct effects of Cd on bone have increasingly come into focus supporting them as critical effects. It is, so, imperative to identify Cd model(s) of action, which will provide new insight in both: a) Cd and/or Cd-based nanoparticles toxicity and b) develop new forms of treatment in case of Cd intoxication.

1.2. Cadmium and Cell Biology

1.2.1. Cadmium as a carcinogen

Cd has been classified as a human carcinogen of category #1 by the International Agency for Research on Cancer and the National Toxicology Program of the USA (21). It has also been suggested that Cd might also be implicated in the pathogenesis of human pancreatic cancer and renal carcinoma (22, 23). Occupational exposure to Cd has been associated with prostate, lung and gastro-intestinal (kidney and pancreas) cancers. Smoking can synergistically increase the carcinogenic effect of Cd (24). The effect of environmental exposure to Cd on cancer incidence had shown an association between risk of cancer and Cd exposure. A possible cause of Cd induced carcinogenicity may rely on its interactions with E-cadherin (a transmembrane Ca(II)-binding glycoprotein playing an important role in cell–cell adhesion). This glycoprotein can also bind Cd to Ca(II)-binding regions, changing the glycoprotein conformation, and so disrupting cell-cell adhesion and eventually becoming a promoter of tumor induction . Cd can also activate cellular protein kinases (protein kinase C), which result in enhanced phosphorylation of various transcription factors which in turn lead to activation of target gene expression (25).

It is well known that Cd also interferes with the biokinetics and biological roles of many essential metals and metalloids such calcium (Ca), magnesium (Mg), sodium (Na), potassium (K), zinc (Zn), copper (Cu), iron (Fe), manganese (Mn), selenium (Se), molybdenum (Mo), trivalent chromium [Cr(III)], cobalt (Co), boron (B), among others (26). However, the exact mechanisms involved in these interactions have not been entirely identified. Cd and Zn interactions are probably among the most recognized metal-metal interactions. This interaction was strongly supported by data that correlated the increasing exposure of the population to environmental Cd and an increase of Zn deficiency (15). Zn is a co-factor in innumerous enzymes and regulatory proteins, including enzymes for synthesis and repair of DNA and RNA. Moreover, because Cd and Zn have similar characteristics - both belong to the group 12 of the periodic table and form tetrahedral complexes, they compete for the same binding sites and/or ligands in biological systems. This interaction causes impairment in Zn homeostasis and may have serious consequences on cell growth, development, and their functions.

1.2.2. **Cadmium and oxidative stress**

Although the toxic mechanisms of Cd are not fully understood, it's known that Cd acts intracellulary mainly by free radical-induced damage, particularly to the lungs, kidneys, bone, central nervous system, reproductive organs and heart (27). Moreover, Cd by itself, is unable to generate free radicals directly, indirect formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) involving the superoxide radical, hydroxyl radical and nitric oxide has been reported (22). Other authors also described the generation of H_2O_2, induced by Cd which can be a major source of radicals via Fenton chemistry. Indeed, Prince proposed in 1983 (28) a mechanisms in which Cd can replace Fe and Cu in several cytoplasmic or membrane proteins (e.g., ferritin, apoferritin) thus increasing the pool of unbound Fe and Cu ions, which are capable of creating oxidative stress via Fenton reactions. These results are also supported by more recent findings on Cd-induced displacements of other cations (29)

Once inside the cell, Cd may also target mitochondria, mostly by binding to protein thiols in mitochondrial membranes. This interaction may influence mitochondrial permeability transition, inhibit electron transport chain, and may lead to ROS generation. Moreover, Cd inhibits mitochondrial complex III, which lead to semiubiquinone accumulation, promoting the transference of electrons to molecular oxygen forming superoxide anions. These Cd-mitochondria interactions are in general present in both plant and animal cells, and it is evident that this is an important source for Cd generated ROS in the cell (30).

2. OXIDATIVE STRESS

Oxidative stress has been defined as a disturbance in the balance between the production of oxidant species (free radicals) and antioxidant defenses, which may lead to tissue injury (31). Free radicals are formed in large amounts as an unavoidable by-product of many biochemical processes and in some instances, deliberately (e.g. as it happens in activated neutrophils). In addition, free radicals may be generated in the body in response to electromagnetic and ionizing radiation like X-rays and by gamma rays from the environment or acquired directly as oxidizing pollutants such as ozone and nitrogen dioxide. At the physiological level they can be produced by metal-catalyzed reactions, by neutrophils and macrophages activation during inflammation and by mitochondria (32).

As result of free radical generalized attack of cell components, complex antioxidant defense mechanisms have evolved to protect body tissues. Despite the presence of this antioxidant system, oxidative damage accumulates during the life cycle. Its accumulation in DNA, proteins and lipids play a key role in the development of age-dependent diseases such as cancer, arteriosclerosis, arthritis, neurodegenerative disorders, among other conditions. ROS are also implicated in several signaling pathways, which are well harmonized with cell antioxidant network (33)

2.1. Free Radicals

Free radicals are any chemical species with independent existence that contains unpaired electrons (32). These electrons increase the chemical reactivity of an atom or molecule. The most common examples of free radicals are the hydroxyl radical ($^{\bullet}OH$), superoxide anion ($^{\bullet}O_2^-$), transition metals such as iron and copper, nitric oxide ($^{\bullet}NO$) (32).

Free radicals can be produced by different biochemical processes within the body including: reduction of molecular oxygen during aerobic respiration yielding superoxide and hydroxyl radicals in mitochondrial electron transport chain; as a by-products of chemistry such as oxidation of catecholamines and activation of the arachidonic acid cascade product electrons, which can reduce molecular oxygen to superoxide; in cytochrome P450 enzymes metabolism; in, e.g., vascular endothelium that produce nitric oxide; or in inflammatory cell activation. In addition, free radicals can be produced

physiologically in response to environmental stress agents, e.g. metal ions, electromagnetic radiation (13, 23).

The presence of unpaired electrons in free radicals confers them highly reactivity, although this varies from radical to radical. Free radicals need to achieve a more stable state by acceptance or donation of electrons to other molecules. As most molecules are not free radicals, the majority of reactions will involve non-radical species. Reactions of a radical with a non-radical (all biological macromolecules are possible targets) produce free radical chain reactions with the formation of new radicals, which in turn can react with further macromolecules. Important examples are lipid peroxidation and protein damage, e.g, addition of carbonyl groups. Carbonyl derivatives of amino acid residues render the protein susceptible to proteolysis. From interaction with free radicals, DNA may also suffer changes, namely damage (see 2.3). These can be all symptomatic effects for numerous diseases such as: cancer, cardiovascular, diabetes, atherosclerosis, neurological disorders (Alzheimer's disease and Parkinson's disease) and chronic inflammation, among others. Oxidative species can also lead to apoptosis by the activations of the intrinsic pathway (34). Lipid peroxidation products [e.g., malondialdehyde (MDA), 4-hydroxynonenal (HNE)] are potent mutagens and have been reported as modulators of signal pathways related to proliferation and apoptosis, processes implicated in carcinogenesis (35)

The hydroxyl radical, the most potent oxidant known, has an extremely short half-life, and the capability to attack most biological molecules resulting in the propagation of free radical chain reactions. It can be generated through a variety of mechanisms. Ionising radiation, which causes decomposition of H_2O, resulting in the formation of $^{\bullet}OH$ and hydrogen atoms, is also generated by photolytic decomposition of alkylhydroperoxides, or by Fenton chemistry using metal as reducing agents. The metal-catalyzed breakdown of hydrogen peroxide, according to the general Fenton reaction (see equation 1, where M^{n+} is a metal) occurs when M^{n+} is Fe, Cu or Cr. Although other metal can catalyze this reaction, these are the most prevalent catalyzers

$$Eq.\,1: M^{n+} + H_2O_2 \rightarrow M^{(n+1)} + \ ^{\cdot}OH + OH^{-}.$$

Superoxide anion is formed when oxygen accepts an electron. Superoxide anion can act as a reducing agent of iron complexes (e.g., cytochrome c) by the Waber-Weiss

reaction and it is likely to be an important source of hydroxyl radicals and hydrogen peroxide (23,36). On the other hand, the redox inactive metals, such as Cd, arsenic (As) and lead (Pb) can promote oxidative stress indirectly, either by alteration of signaling pathways or disrupting anti-oxidant homeostasis. Indeed recent notes describe the direct formation of H_2O_2 by arsenic under physiological condition and depletion of glutathione reduced by Cd (for review see Jomova 2011 (13)).

2.2. Anti-Oxidant Defenses

Antioxidant is any substance that when present at low concentrations (compared with those of the oxidizable substrate) considerably delays or inhibits its oxidation (33). Cell antioxidative defense can be categorized as enzymatic, non-enzymatic and extracellular mechanisms. The enzymatic defense is mediated by a group of enzymes which are capable of metabolize ROS and ROS precursors (e.g. H_2O_2). In the same way, non-enzymatic defenses are responsible for an identical process, but without the aid of enzymes, instead, are used several molecules that can donate electrons to radical species or by processes who diminish ROS production (33, 37, 38).

2.2.1. Enzymatic defenses

Cellular antioxidant defenses include the superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) enzymes (33). These enzymes act in chain to achieve the scavenger of free radicals. SOD isoforms are differently distributed among intra and extra cellular spaces. These isoenzymes are capable of catalyzing the dismutation of superoxide anion to hydrogen peroxide and molecular oxygen (equation 9)(39).

$$Eq.2: 2O_2^{\cdot -} + 2H^+ \xrightarrow{SOD} 2H_2O_2 + O_2$$

Although, H_2O_2 is a weaker oxidant than superoxide anion it is relatively stable and can easily diffuse across biological membranes entering in cellular compartments. Two enzymes can breakdown H_2O_2, GPx and CAT. The first is present in cytosol and mitochondria, and has a major role in the breakdown of most H_2O_2 generated by SOD. In

the reduction of H_2O_2, as illustrated by equation 3, GPx uses as antioxidant the reduced glutathione (GSH) generating H_2O and oxidized glutathione (GSSG)(40).

$$Eq.3: 2GSH + ROOH \xrightarrow{GPx} GSSG + ROH + H_2O$$

CAT can also breakdown H_2O_2 to molecular oxygen. It is localized in the peroxisomes, and is mostly responsible for the removal of H_2O_2 when it is present in higher concentration in tissue (equation 4) (41).

$$Eq.4: H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

When these enzymatic systems work together, they efficiently maintain the counterbalance oxidant species in cells and tissues.

2.2.2. **Non-enzymatic defenses**

Cells contain non-enzymatic systems capable of contain free radicals insults. Molecules such as vitamin E, coenzyme Q, β-carotene and other carotenes are present within cell membranes. Also vitamin C (L-ascorbic acid) and GSH are molecules present in cytosol (38). These non-enzymatic molecules act as antioxidants and can sustain oxidative stress. Furthermore, tissues are also protected against free radicals by several binding proteins (transferrin, lactoferrin, ceruloplasmin, haptoglobins, hemopexin and albumin) which can sequester metals and keep it in a nonreactive state (23). Indeed the mechanism of metal-induced free radicals formation is tightly influenced by the action of cellular antioxidants. These proteins are highly inducible by metals and oxidants and thus, are critical in protection against exogenous oxidants, as is the case of Cd (42). Moreover, many low-molecular weight antioxidants such as ascorbic acid, alpha-tocopherol, glutathione (GSH), carotenoids, flavonoids, and other antioxidants, are capable of chelating metal ions reducing thus their catalytic activity to form ROS (13).

2.2.3. **Others defenses**

Besides the above reported antioxidative systems, cells evolved complementary mechanisms to regulate ROS production, namely the metabolic uncoupling of mitochondria. The short-cut of the mitochondrial electron chain by uncoupling proteins

(UCP) decrease the ROS formation where the reduction power of oxidative metabolism is transferred to heath production and ROS-formation control (42).

2.3. Oxidative Damage to Biomolecules

2.3.1. DNA damage

Reactive oxygen species are formed through a variety of events and pathways and can react with almost every molecule in cell. The hydroxyl radical is known to directly react with all components of the DNA molecule. It can damage purine and pyrimidine bases and also the deoxyribose backbone (43). Permanent modification of genetic material represents the first step involved in mutagenesis, carcinogenesis and ageing. In fact, is well established, in various cancer tissues that free radical-mediated DNA damage had occurred. ROS-induced DNA damage can involve single- or double stranded DNA breaks, purine, pyrimidine, or deoxyribose modifications, and DNA cross-links. DNA damage can result either in arrest or induction of transcription, induction of signal transduction pathways, replication errors and genomic instability, all associated to carcinogenesis (44).

A good biomarker for DNA oxidative stress and a potential biomarker of carcinogenesis is 8-hydroxyguanine (8-OH-G or 8-oxo-G). This oxidized DNA product is important because it is easily produced and also is mutagenic and carcinogenic. Several studies have reported 8-OH-G link with oxidative damage (45). Other methods for measuring oxidative DNA damage may involve for example, enzymatic digestion of DNA; the acidic hydrolysis of DNA (46).

Mutations and altered expression in mitochondrial genes encoding for respiratory complexes, and in the hypervariable regions of $_{mt}$DNA were also identified in several cancers (47). $_{mt}$DNA is close ROS formation, is not protect by histones and has limited repairing capability, for all of that it is an easy target to ROS (48).

2.3.2. Lipid peroxidation

All cellular membranes are sensitive to oxidative attack namely those membranes with polyunsaturated fatty acids (PUFA) in its composition. The process of lipid peroxidation consists of three stages: initiation, propagation and termination. The peroxyl radicals (ROO•) after formation can be arranged via a cyclization reaction to

endoperoxides with the final product of the peroxidation process being malondialdehyde (MDA) (49). The major aldehyde product of lipid peroxidation other than malondialdehyde is 4-hydroxy-2-nonenal (HNE). MDA is mutagenic in bacterial and mammalian cells and carcinogenic in rats (50). HNE is weakly mutagenic but is the major toxic product of lipid peroxidation. It also has a powerful effect on signal transduction pathways. Moreover, lipid peroxidation products can interact with DNA, creating adducts that can lead to DNA–DNA interstrand crosslinks or DNA–protein crosslinks (50). MDA also exerts direct modifications on DNA strands. All of these modifications can be deleterious to cells either being genotoxic or carcinogenic. (23).

2.3.3. **Protein oxidation**

Proteins can also be a target for ROS oxidation. *In vivo* this is achieved in the presence of transition metals capable of entering in Fenton reactions (equation 1). Metal-catalyzed damages of proteins involve oxidative scission, loss of histidine residues, bityrosine crosslinks and introduction of carbonyl groups (51).

The hydroxyl radical has the ability to remove hydrogen atom from the protein polypeptide backbone forming a carbon centered radical, which can readily react with molecular oxygen to form peroxyl radicals (52). Protein damage is likely to be repairable and is a known non-lethal event for a cell. The side chains of all amino acid residues of proteins are susceptible to oxidation by ionising radiation and by the action of ROS/RNS. However, proline, histidine, arginine, lysine and cysteine residues are sensitive to oxidation by redox metals and may be preferable protein sites of metal binding. This protein-iron complex can react with H_2O_2, forming hydroxyl radical on site which can rapidly promote oxidation process (53)

Nitrosylation reactions can also attack proteins. The reaction of nitric oxide with superoxide radical results in the formation of the highly toxic peroxinitrite anion which can nitrosate cystein, nitrate tyrosine and tryptophan and oxidize methionine to methionine sulphoxide. However, the process of modification of proteins by peroxynitrite is strongly inhibited by physiological concentration of carbon dioxide (23). Even though the nitration of tyrosine is a irreversible process, and can interact in phosphorylation and adenylylation process which are important in regulatory pathways (23).

The accumulation of oxidized proteins in living systems can be induced by several factors acting isolately or in combination. As consequence of these protein- ROS interactions, inter- and intra-protein cross-linkages occur. Moreover, protein carbonyl groups are generated. The concentration of protein carbonyl groups is considered a good biomarker of ROS mediated protein oxidation.

2.4. ROS and Cancer

Carcinogenesis is a complex multi-sequence process of cell conversion from a healthy stage to pre-cancerous and, later, cancer stages. Moreover, the process of carcinogenesis consists of multiple distinctive stages, each one characterized by different underlying mechanisms. The "initiation–promotion–progression" model of carcinogenesis mechanism is based on the equilibrium between cell proliferation and cell death (22). Cell proliferation is upregulated by protein p53 which plays a primordial role in it, its checks the DNA integrity during cell cycle (54), and triggers mechanisms of DNA repair, for instance to eliminate the oxidized DNA bases that cause mutations. Moreover, at the same time if the cell damage is too great, it can also trigger cell death by apoptosis. So, there is a subtle regulatory system consisting of pro-apoptotic and anti-apoptotic factors controlling cell fate. Cancer development is characterized by cumulative action of multiple events occurring in a single cell and can be described by the three stages: initiation, promotion and progression. ROS can act in all these stages of carcinogenesis through modifications on transductions or signaling pathways and also on cellular events. Similarly, several chemicals may act as carcinogens by having genotoxic effects or by non-DNA or indirect-DNA mechanisms.

ROS can interact with DNA in the initiation process, causing non-lethal mutation that can pass cell cycle checkpoints. The dual role of oxidative stress is expressive in both pathways involving cell-cycle and apoptosis deregulation. Although excess of oxidative stress can be cytotoxic and halt cell proliferation and induce apoptosis or necrosis, small increments can promote cell proliferation inducing tumor growth. Many tumor promoters have an inhibiting effect on cellular antioxidant defenses (55). Furthermore, ROS and several metal can promote abnormalities in growth factor receptors, and by is one or acting through other systems alter Ca^{2+} homeostasis, leading to deregulation of many signaling pathways that controls cell proliferation or apoptosis (55).

The stage of tumor progression involves cellular and molecular changes and is characterized by accumulation of additional genetic damage, leading to the transition of the cell from benign to malignant. One characteristic of this stage is the angiogenesis, which promotes the blood supply to tumor cells. Moreover, ROS implicated in tumor angiogenesis and may regulate tumor invasiveness by modulating the expression (42, 56, 57).

2.4.1. **Cadmium, ROS and apoptosis**

By the above exposed it is highly probable that ROS-inducing chemicals may also modulate cell events, in particular cell cycle and apoptosis, traditionally dysfunctional in tumor cells (58). Cd does not *per se* form free radicals directly, but cells exposed to Cd^{2+} clearly show elevated levels of ROS (59). Some explanations may relay on the fact that Cd is highly reactive to protein thiols, depleting the antioxidant cell battery. So, Cd can induce responses leading to oxidative stress, but it is still unknown how Cd affects the apoptosis pathways. Apoptosis is currently evaluated by the Annexin V. This method is based on a property of protein localized predominantly in the inner side of the membrane, the phosphatidylserine, which during the first stages of apoptosis is exposed to the outer side. Other methods such as TUNEL, caspases expression may also be used (60-62), but this will not be subject of this dissertation.

3. CADMIUM AND OSTEOBLAST METABOLISM

Bone tissue, is the major structural and supportive connective tissue of the body forming the body skeleton. Furthermore, the bone matrix is formed by the mineralization of several elements, namely calcium, magnesium and phosphate in a matrix of type-I collagen, which form hydroxyhapatite. This forms a rigid but flexible tissue capable of support mechanical charges and by that support the entire body. In addition, it can protect vital organs (e.g., brain, heart and lungs) and it is also important in the body movement and locomotion.

Although is rigid and mineral composition, bone is a dynamic tissue capable of remodeling. Osteoblasts are the cells responsible for the formation of bone; they synthesize all the constituents of the bone matrix and direct its subsequent mineralization. Once a phase of active bone formation is completed the osteoblasts do not become senescent, but

instead re-differentiate into one of two major cell types: osteocytes and osteoclasts. Both play a major role in the regulation of calcium homeostasis and bone remodeling. Moreover, because bone mass is maintained constant by the balance between osteoclastic bone resorption and osteoblastic bone formation, alterations in osteoblast proliferation and differentiation may disturb the equilibrium of bone remodeling and calcium homeostasis (63,64).

Furthermore, exposure to Cd has been associated with the alteration of bone metabolism and the development of osteoporosis among other alterations. Little information is available reporting the Cd accumulation and direct/indirect effects on osteoblast cells. Clarification of Cd induced cytotoxicity in osteoblasts will therefore contribute to better understand diseases promoted by Cd intoxication like osteoporosis (65) or, in particular, tumor (58, 66).

3.1. Osteoblast *in vitro* Culture

Osteoblasts are central to the process of bone remodeling and are involved in many bone diseases. Primary osteoblasts obtained directly from patients are the idyllic cells to in vitro studies. However, the use of primary osteoblasts in vitro is limited because of difficulty in obtaining sufficient bone fragments for harvesting cells. Furthermore, primary osteoblasts are difficult to culture in vitro, have a finite life-span and also can lose their phenotype as the number of passages increases (67).

One way to bypass that problem is to study osteosarcoma-derived cell lines which are highly proliferative. Moreover, cell lines derived from human cancers have been crucial to building our understanding of the molecular pathophysiology of cancer and its treatment, and have been routinely used as a model for osteoblasts to characterize their interaction with various biomaterials and are widely used in cytotoxic studies. Equally, they form an in vitro model system for rational drug discovery and development because they are easy to maintain and manipulate in vitro and in animal models (68).

Many osteoblast-like cell lines have been developed. Osteosarcoma derived cell lines are relatively easy to culture and have a high rate of proliferation and long life-span. The MG-63 osteoblast-like cells line used in this study was originally isolated from a human osteosarcoma. MG-63 cell line is relatively immature osteoblasts that have been

well characterized, it presents an anomalous karyotype, with multiple triploidy, however they show several similarities with isolated human bone-derived cells (69, 72). Indeed, they are widely used for testing biomaterials and in toxicological screening (70, 71). Moreover, MG-63 cells present biomarkers exclusive of human osteoblasts, including alkaline phosphatase, bone sialoprotein, osteonectin, osteopontin, and growth factors (e.g., bone morphogenetic protein). Altogether, MG-63 cells are suitable to serve as a model of human osteoblast cells (69,73).

Objectives

The main aim of the present dissertation was to evaluate putative cytotoxic effects of Cd in bone cells. As described in the introduction, a particularly sensitive cascade in the cell is the one involving oxidative stress unbalances. This issue still remains fuzzy in what concerns Cd-cell interactions. A complete elucidation of oxidative stress deregulation by Cd will help in deciphering later and depended mechanisms such as apoptosis and cell cycle, and ultimately aspects related with carcinogenesis.

To achieve this, it was used the cell line MG-63, a line with rapid growth and retaining osteoblast like characteristics, and one of the most widely used cell lines in these kind of assays in bone studies in vitro.

These cells were exposed to increasing concentrations of Cd, and some of the most important enzymes of oxidative stress were measured. Also Cd-induced apoptosis was evaluated by annexinV. An integrated analyse of these responses will provide a more complete insight of the effects of Cd in osteoblasts.

Material and Methods

4. MATERIAL

The cell line MG-63 was courtesy of INEB (Instituto de Engenharia Biomédica-Porto, Portugal). GIBCO® Culture medium α-MEM GlutaMAX™, Trypsin-EDTA 0.25%, Fungizone and Peniciline/Streptomicine were purchased from Life Technologies (Carlsbad, US-CA). SOD Assay Kit and Total Glutathione Assay Kit were obtained from Sigma-Aldrich (St. Louis, US-MO). BD Pharmingen™ Annexin V: FITC Apoptosis Detection Kit was bought to Bencton Dickinson and Company (Franklin Lakes, US-NJ).

All other reagents were purchased from Sigma-Aldrich (St. Louis, US-MO) at highest grade available. All solutions were prepared using ultrapure water obtained in a MILI-Q System from Millipore (Billerica, US-MA).

5. METHODS

5.1. Cell Culture

The MG-63 cell line was cultured in α-MEM GlutaMAX™ medium and incubated with controlled atmosphere at 37 °C and 5% CO_2. Cells were subcultured once a week. The cell culture was maintained in 75 cm^2 standard culture flasks and the medium consisted of 10 ml of α-Minimal Essential Medium with GlutaMAX™ (α-MEM) supplemented with 10% (v/v) of Fetal Bovine Serum (FBS), 100 U/mL of Peniciline/Streptomicine and 2.50 µg/mL of Fungizone (final concentrations). The passages were done before the cells reach full confluence.

5.1.1. Harvesting cells

The standard process for cell harvesting was performed by addition of trypsine, a proteolitic enzyme. Cells were first washed twice with Phosphate Buffer Saline without Ca^{2+}/Mg^{2+} (PBS), and then incubated with trypsine (1 mL/25 cm^2) for 5-10 min at 37 °C. When cells were detached, the trypsine was deactivated with growth medium with FBS and the cells were resuspended to be counted by using a hemocytometer. For subculture procedure, cells were seeded at 10^5 cell/mL in 10 mL of supplemented medium.

5.1.2. Cell exposure

All assays were performed within twenty passages from defrosting. For all the assays the procedure to exposure remains mostly the same. Mainly, cells were plated and left 24 hours to adhere and when the cells reached 50-60% of confluence, the seeding medium was then removed and substituted by cadmium chloride dissolved in supplemented medium at final concentrations of 20 and 50 µM. Cells were left to growth for 24 and 48 hours and then harvested. Depending of the particular assay cells undergone slightly different seeding and harvesting methods.

5.2. Protein Quantification

The Bradford assay, is a colorimetric method to quantify the protein content of samples. This method is based in the binding of the dye Coomassie Brilliant Blue G-250

(Bradford Reagent) to protein which causes a shift in it absorption from 465 nm to 595 nm, the amount of absorption is proportional to the quantity of protein (74).

In brief, 5 µL of each cell extract was incubated in 250 µL of Bradford Reagent left to incubate in dark for 10 min and then the absorbance read at 595 nm in a microplate reader (Synergy HT from BioTek®). Concentrations of unknown samples were extrapolated from a standard curve performed with known concentration of bovine serum albumin (BSA).

5.3. Cell Viability

Cell viability was measured by the MTT assay as described by Twentyman (75) with some modifications. MTT assay relies on the capability of viable cells to reduce a tetrazolium salt (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide-MTT) to an intercellular purple formazan which can be solubilized and quantified spectrophotometrically. The signal produced is linear to the quantity of viable cells.

To verify the interaction of Cd on cell viability, cells were seeded in a 96 well plate at the concentration of 10^5 cells/mL. Cells were left 24 hours to adhere and then were exposed to 20 and 50 µM of Cd in growth medium. After time exposure (24/48 hours), cells were incubated with 50 µL of MTT (1 mg/mL in sterile PBS) for 4 hours at 37 °C. The medium was removed and 150 µL of DMSO (Dimethyl sulfoxide) were added, then cells were stirred in dark for two hours in order to dissolve all formazan crystals. After this time, at the absorbance for each well was measured at 570 nm with a microplate reader (Synergy™ HT Multi-Mode from BioTeK®). The data was expressed by the ratio of absorbance between exposed and non-exposed cells in percentage.

5.4. Enzymatic Parameters

To assay all enzymes, cells were seeded in 100 mm Corning® culture dishes and exposed to Cd as described above in 5.1.2. To prepare homogenates the method described by Quick (76) was followed with some modification. Briefly, the culture medium was removed and 2 mL of cold phosphate buffer (5 mM, pH 7.4) with 0.5 mM of EDTA was added and cells were frozen to disrupt membranes. After thawing, the cultures were scraped and sonicated 3 times for 30s over ice. Finally, to clear cellular debris the

homogenates were centrifuged at 12,000 xg for 15 min at 4 °C. The pellet was discarded and the supernatants were aliquoted to separated vials, one for each enzyme and other for protein quantification. All samples were stored at -80 °C until assayed.

5.4.1. **Superoxide Dismutase**

Superoxide Dismutase (SOD) catalyzes the dismutation of superoxide anion $O_2^{\bullet-}$ to molecular oxygen and oxygen peroxide (H_2O_2). At present, three distinct isoforms of SOD have been identified in mammals, CuZn-SOD, Mn-SOD and EC-SOD which differ in their structure and location. They are the first line of defense against oxidative stress namely by acting as a superoxide anion radical scavenger (39).

Total SOD activity was measured by using Sigma-Aldrich® *SOD-assay kit*. This kit uses a coupled reaction in which SOD competes with xantine oxidase (XO) by the superoxide anion in the reduction of a water soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl)- 3-(4-nitrophenyl)-5-(2,4-disulfophenyl)- 2H-tetrazolium,monosodium salt). The reaction was performed in accordance to kit instructions. It consisted of the addition of 20 μL of sample to 200 μL of a buffer with WST-1. The reaction started with the addition of 20 μL of xantine-oxidase (XO) solution. The reaction was carried out in a 96 well plate, and the absorbance variation was read in a microplate reader (Synergy™ HT Multi-Mode from BioTeK®) in kinetic mode at 440 nm for 5 min. The percentage of inhibition was calculated by the following equation:

$$Eq.5 \quad XO\ \%\ inhibition\ = \frac{(S1 - S3) - (SS - S2)}{(S1 - S3)} \times 100$$

The reaction was always performed with a “blank well” to calculate xantine-oxidase maximum rate (S1). Several other “blank wells” were attributed to account with non specific reduction of WST-1 and other sources of superoxide anion, namely a well without XO and sample (S3), and other only without XO (S2). All results were normalized by samples protein concentration and one unit of SOD activity was considered as the quantity of enzyme which gives a 50% inhibition of the reduction of WST-1.

5.4.2. **Catalase**

Catalase exerts a dual function on cell physiology, a catalytic function with the decomposition of hydrogen peroxide (H_2O_2) to water and molecular oxygen and a peroxidic activity, oxidation of hydrogen donors like methanol and ethanol (41, 47)

Catalase activity was directly measured by the decay in absorbance of H_2O_2 at 240 nm. The difference in absorbance at 240 nm per unit of time is the measure of its activity. This assay was performed accordingly to Aebi (41) with few modifications. The reaction was carried out in a 1.5 mL quartz cuvette at 25 °C. The reaction started with the addition of H_2O_2 (12,5 mM final concentration) to 50 µL of sample in 50 mM phosphate buffer pH 7.0 all mixed by inversion. The absorbance reading was taken against a "Blank" composed of only buffer with sample. The absorbance decay was followed and one unit of catalase activity was expressed by the number of nmoles of H_2O_2 consumed per minute (41).

5.4.3. **Glutathione Reductase**

GR plays an important role in cell antioxidant state by producing antioxidant species. GR catalyzes the reduction of glutathione disulfide (GSSG) produced by GPx activity, to the glutathione reduced form (GSH) using NAPDH as its source of reducing equivalents. GSH reduced power can again be used by glutathione peroxidase (GPx) in reduction of peroxides. These reaction produce GSSG which is then recycled to GSH by GR (Equation 6).

$$Eq.6: \ GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+$$

GR activity was measured directly by the decay in absorbance of NADPH at 340 nm in a microplate reader accordingly to Dringen (77) with slightly modifications. The reaction was carried in a phosphate buffer 100 mM with EDTA 1 mM at 7.0 pH and 25 °C. The reaction was started by the addition of GSSG solution in 1:1 to the mixture of NADPH and sample in a 96 wells microplate plate. The final concentrations were GSSG 1 mM and 0.2 mM of NADPH in a final volume of 320 µL. In parallel with samples measurements, it was performed a calibration curve with standard GR to extrapolate the sample activity.

Finally, the GR's specific activity was expressed as a ratio of its activity and the protein content of the samples.

5.4.4. **Total Glutathione quantification**

Glutathione as been described earlier have an important role in antioxidant status. Briefly GSH serves as a co-substrate to glutathione transferase in xenobiotics detoxification and as an electron donor to GPx having an antioxidant role (78).

The quantification of total glutathione was done with *The Glutathione Assay Kit* from Sigma-Aldrich®. This utilizes an enzymatic recycling method, which uses glutathione reductase to quantify GSH/GSSG content of the sample. This method relies on the capability of DTNB (5.5'-dithio-*bis*-2-nitrobenzoic acid or Ellman's reagent) to react with GSH producing a yellow compound, 5-thio-2-nitrobenzoic acid (TNB). GR recycles the glutathione which is then reused in the reaction. The rate of TNB production is directly proportional to this recycling reaction which in turn is directly proportional to the concentration of GSH in the sample. The TNB formation is followed at 412 nm, and the total glutathione content is extrapolated with the aid of a calibration curve with known GSH concentrations.

For this assay, kit instructions were followed. Briefly cells were seeded in 150 mm Corning® culture dishes in order to achieve the harvest of at least 10^6 cells. Cd exposure was performed was described in 5.1.2. Cells were harvested by scrapping from plates in PBS buffer and concentrated by centrifugation at 600 xg. The supernatant was discarded and added 3 volumes of 5% SSA (5-Sulfosalicylic Acid) after homogenization two freeze and thaw cycles were performed. Finally the homogenates centrifuged at 10000 x g for 10 min at 4 °C, to clear cellular debris, pellets were discarded and total glutathione content were measured in supernatant. To quantify glutathione content, 10 µL of each sample were added to one well of a 96 well plate in triplicate, followed by the addition of a buffer containing DTNB, GR and finally to start the reaction a NADPH solution (all supplied by the Kit). The reactions were measured in kinetic mode at 412 nm in a multiplate reader (Synergy™ HT Multi-Mode from BioTeK®). Glutathione concentrations of all samples were extrapolated from a standard curve with known concentrations of reduced glutathione diluted in 5% SSA performed at same time of the assay.

5.5. Apoptose

5.5.1. Annexin V assay

Apoptosis or programmed cell death is a normal physiologic process. The apoptotic program is characterized by morphologic features, including loss of plasma membrane asymmetry and attachment, nucleus condensation, and cleavage of DNA. Loss of plasma membrane is one of the earliest features. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, exposing PS externally.

Annexin V is a phospholipid-binding protein that has a high affinity for PS. In this assay Annexin V is used conjugated with fluorescein isothiocyanate (FITC) as a sensitive probe to PS. The cells which are undergoing apoptosis can then be access by flow cytometry. Since externalization of PS occurs in the earlier stages of apoptosis, FITC Annexin V staining can identify apoptosis at an earlier stage than other assays, namely, those based on nuclear changes.

Apoptosis was measured by flow cytometry using *FITC Annexin V Apoptosis Detection Kit* from BD Pharmingem™. Briefly this kit uses a double staining with Annexin V-FITC and propidium iodide (PI). The double staining with PI, which is excluded by intact plasma membranes, allows the differentiation of early apoptotic (Annexin V-FITC positive/PI negative cells) from late apoptotic (Annexin V-FITC positive/PI positive).

For this assay cells were harvested with trypsine and centrifuged at 300x g twice in cold PBS. Finally cells were resuspended in "*1x binding buffer"* at 10^5 cells/mL, 100 μL of sample was used and added 5 μL of FITC-Annexin V and PI to each sample. Samples were then left in dark for 15 min, and after this time 400 μL of "*1x Binding Buffer"* was added and samples analyzed by flow cytometry.

5.6. Data and Statistical Analysis

For all assays were performed at least three independent experiments. The statistical analysis was performed in the software SigmaPlot version 11. All analysis of variance were performed with one-way ANOVA, followed by Holm-Sidak to test differences between groups and the control exposure. It was considered that results are statistically significant when $p<0.05$. All data is expressed as mean ± standard deviation (SD) from mean.

Results

6. Global Screening Of Cell Cultures Exposed To Cadmium

Control MG-63 cells presented a good proliferation, with total confluence being achieved after 4 days. At the inverted microscope (Nikon® Eclipse TS100), cells had typical fibroblastic aspect. Cultures treated with Cd showed some morphological changes such as cell enlargement, and loss of adherence. The cell confluence for all Cd exposures was less than the observed in control. In the highest concentration it could also be seen frequently floating cells (see Figure 1)

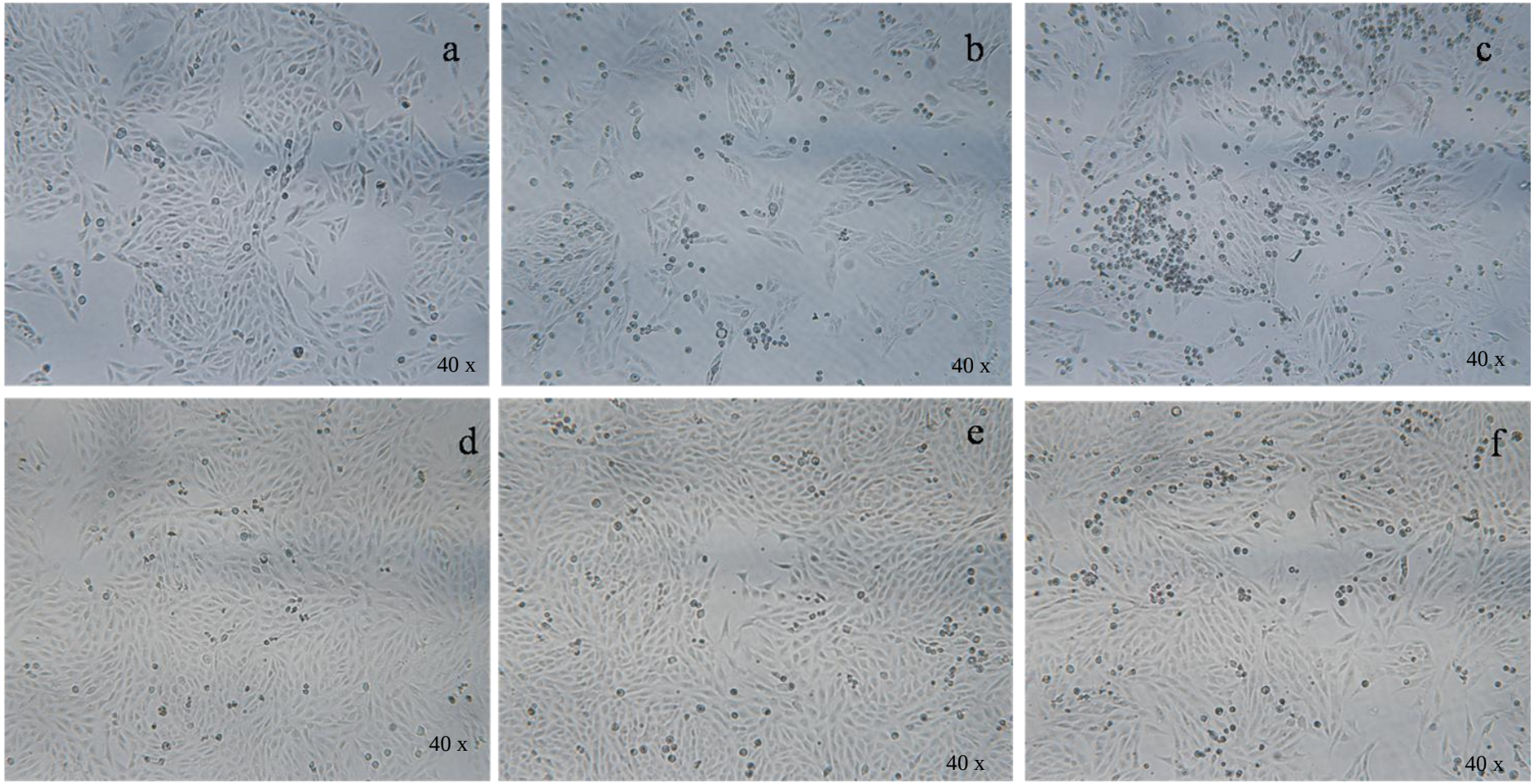

Figure 1-General appearance of MG-63 cell line after Cd exposure. a-b-c- 24 hours exposures, control, 20 and 50 µM respectively; d-e-f- 48 hours exposures, control, 20 and 50 µM respectively

7. VIABILITY ASSAY

The MTT assay was used to asses if Cd could modify cell viability of MG-63 cell line. Data show that Cd induced a dose-response inhibition of cell viability of MG-63 cell line at both times (Figure 2 and Figure 3). At low Cd concentrations of Cd, it was observed a small trend to stimulate cell viability (hormesis effect) despite not significant (Figure 2 and Figure 3). Inhibition

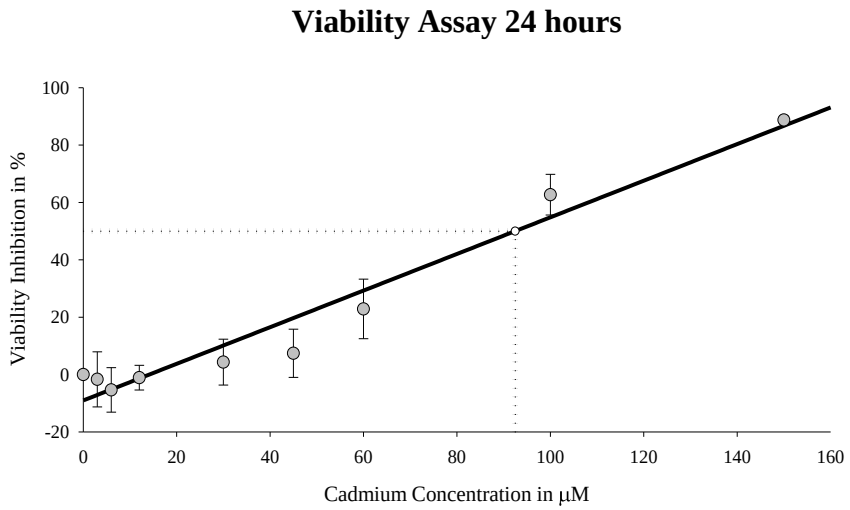

Figure 2- MTT assay for 24 hours. Data expressed as a percentage of control. Values from 5 independent experiments ± SD

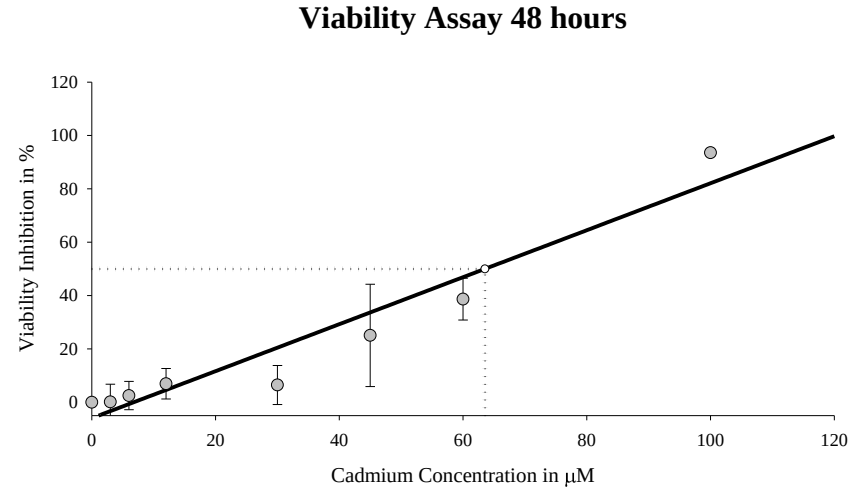

Figure 3-MTT assay for 48 hours. Data expressed as a percentage of control. Values from 5 independent experiments ± SD

A Pearson correlation demonstrates that the relation between Cd concentration and inhibition of cell viability is strong and positive for both periods of the experiment. Namely, the coefficient of correlation for 24 hours is 0.919 ($p<0.0001$), and for 48 hours is 0.891 ($p<0.0001$). With this strong correlation, the concentration of Cd that reduces in half the viability of MG-63, the lethal dose (LD_{50}), could be calculated for both times. Thus, the LD_{50} for Cd exposure in MG-63 cell line is 91 μM, for 24 hours and 64 μM for 48 hours. Based on these results, we chose the sublethal doses of Cd, 20 and 50 μM, for further studies.

8. ENZYMATIC ASSAYS

8.1. Superoxide dismutase activity

The SOD activity shows an increasing trend, with Cd concentration, after 24 and 48 hours (Figure 4). For the 24 hours exposure those differences are not significantly different but have a slight increase. SOD activity at 48 hours shown the same trend with a significant increase for the 50 μM concentration (Figure 4).

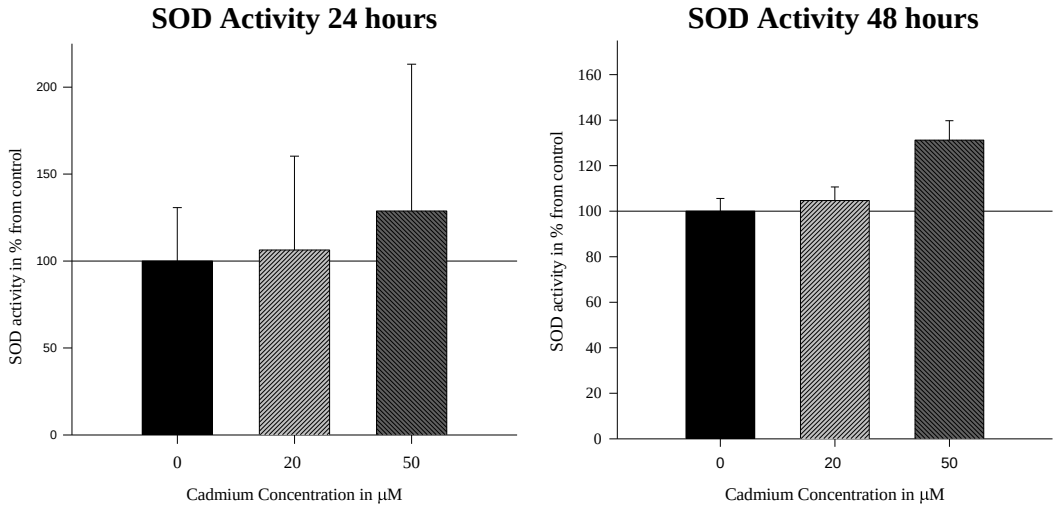

Figure 4-SOD activity for both times expressed as a percentage of control. Values from 4 independent experiments ± SD. * p<0.05

8.2. Catalase activity

CAT activity shows an increasing trend with Cd concentration. Moreover, CAT activity at 24 hours for 50 µM Cd concentration had a significant increase of almost 8 fold. To the 20 µM CAT activity also in 2 fold from control but without statistical significance (Figure 5 – 24 hours). The 48 hours exposure showed the same trend of 24 hours with a continuous and significant increase of near 2 folds of CAT activity for all Cd doses (Figure 5- 48 hours).

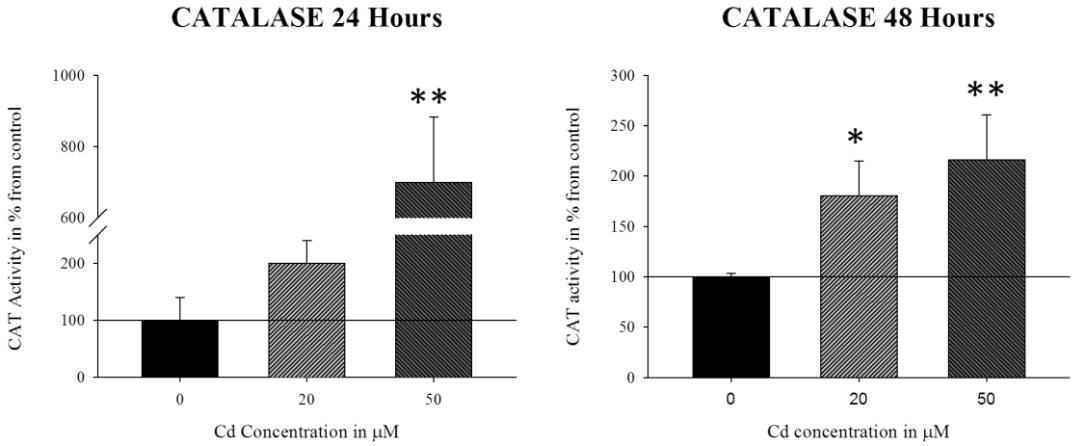

Figure 5 CAT activity for 24 and 48 hours expressed as a percentage of control. Values from 4 independent experiments ± SD (* p<0.05 and ** p<0.001)

8.3. Glutathione Reductase

Cd exposure to MG-63 cell line has a global interaction with GR. The decrease in GR activity is evident in both times. Indeed, both doses led to results significantly different from those of the control. However, only at 24 hours, there was a significant difference ($p<0.05$) among the exposed groups. The observed decrease of GR activity after 48h was not statistically significant (Figure 6).

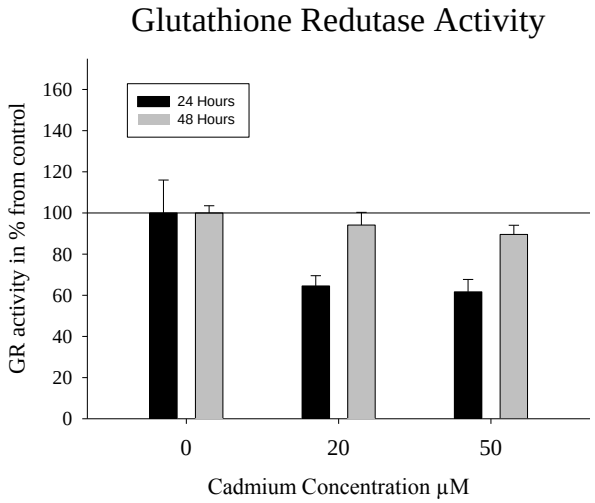

Figure 6-GR activity expressed as percentage of control. Values from 3 independent experiments and expressed in percentage from controls ± SD (* p<0.05)

8.4. Total glutathione content

Cd alters significantly total glutathione content in 24 hours exposure. At this time, both concentrations decrease almost in half its glutathione content (Figure 7-24 hours). However, for the 48 hours exposure there are no differences between control and Cd exposures respecting the glutathione concentration. At this time point, a significant decrease in glutathione concentration was detected in controls between both times (t-test; p<0.001- Figure 7).

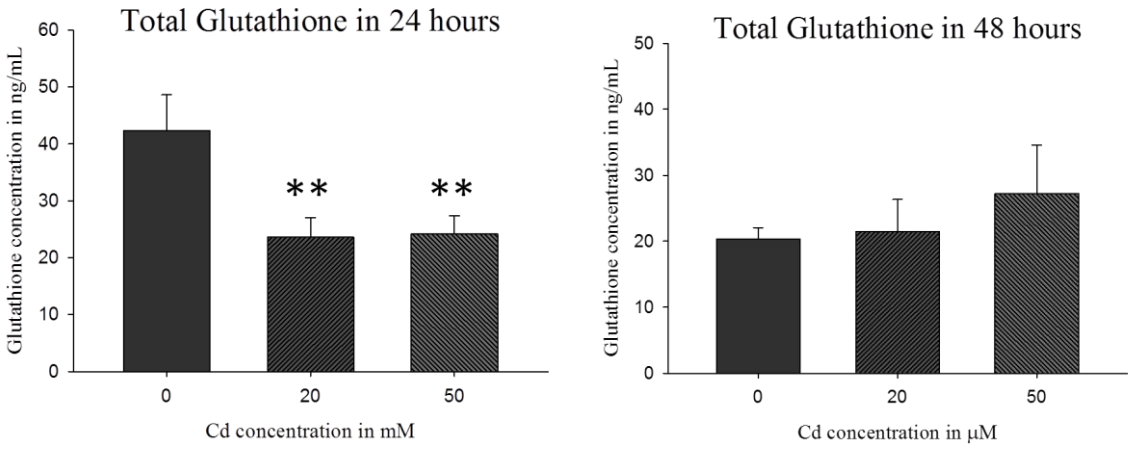

Figure 7-Concentration of glutathione for 24 and 48 hours expressed as a percentage of control. Values from 4 independent experiments ± SD (p<0.001)**

8.5. Apoptosis assay

The results show a significant increase in the early apoptotic event for the 20 µM exposure in 24 hours. Moreover, the late apoptosis and live cell count, although not significant, show differences for the 50 µM exposure, comparatively to the control (Figure 8-24 hours). The 48 hours assay shows us also a difference in apoptosis between conditions. Cd at the highest concentration (50 µM) was significant different from control. Indeed, this Cd concentration increased the number of cells in early and late apoptosis and a decrease in live cell number. For the 20 µM exposure, Cd also increased the number of cells undergoing early apoptosis (Figure 8-48 hours).

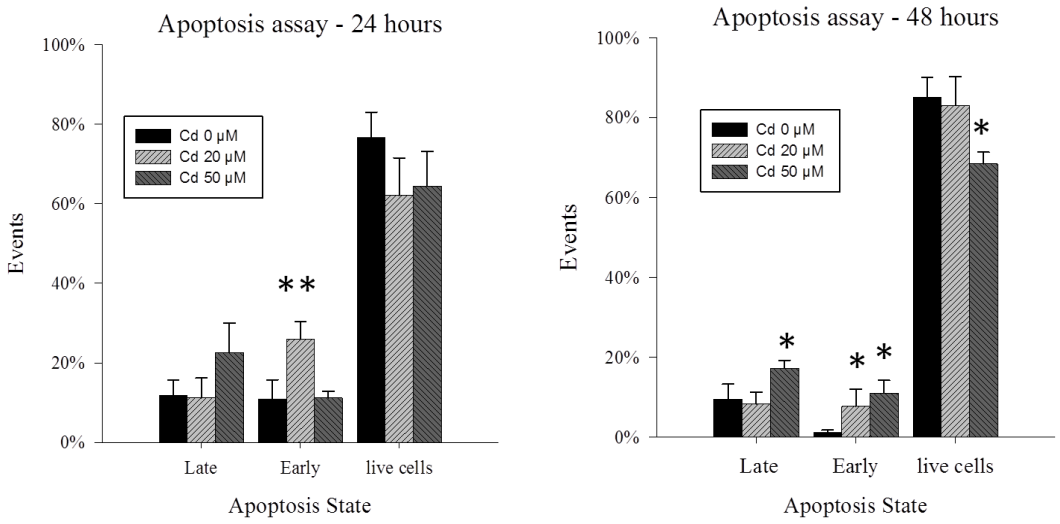

Figure 8-Annexin V-FITC assay for apoptosis assessment. Results for 24 and 48 hours exposures. Values from 4 independent experiments ± SD (* p<0.05 and ** p<0.001)

Discussion

Cd exposure is known to cause disturbances on cell free radicals homeostasis. This is mainly accomplished by disruption of oxidative defenses (13, 22, 30). In MG-63 cell line data of cell viability shows that Cd can induce cell death in a dose-dependent manner and, as expected, this response increases with time exposure. These gradual and time dependent effects quantitatively supported the visual analyses (qualitative) in which cultures with Cd shown a decrease in confluence and a general augmentation in cells detached from the plate (figure 1). In general the data obtained in this dissertation for Cd-induced cell viability is supported by other authors, but a survey of literature shows a strong dependence of genotype/linage in Cd-induced death (79). Indeed, recent studies shown that rat C6 glial cells showed high sensitivity to Cd (20 μM) while the human keratinocytes HaCaT demonstrated high tolerance both reviewed by Nzengue (62).

The small increment in cell viability at very small Cd concentrations supports a putative hormesis effect of Cd. This effect may be justified by a small increment in, ROS levels, which are known to induce cell proliferation (26, 58). Another possible explanation may relay on the possible inhibition of apoptosis regulating proteins (54, 80).

From these results a Cd sublethal dose was chosen to better assess the small changes in cell oxidant defenses. Those doses were chosen to assess small Cd induced alteration before antioxidant defenses are completely overwhelmed, thus allowing us a small scope in Cd-induced alterations. Therefore, Cd concentrations chosen were 20 and 50 μM, both sublethal (< 61 μM at 48 hours).

One of the first lines of antioxidant defenses are the enzymatic antioxidant defenses. Moreover, small disturbances in this system can immediately lead to oxidative stress resulting in several damages to cells (38, 81). The mechanisms by which Cd disrupts oxidative defenses are not fully understood yet, and can vary from tissue to tissue or among lineages (29, 79). Bone tissue relays on redox signaling to its own metabolism, so it is particularly sensitive to alterations on oxidative state (82-84). Indeed the CAT activity was highly increased with both doses and at both times of exposure. This suggests that the cells exposed are subjected to an increase of oxidative stress, namely a surge of H_2O_2. Although, other studies have concluded that Cd decreases CAT activity (85, 86), this is probably due to a different sensitivities in the study model and the Cd concentration used.

With CAT augmentation was also expected a parallel increase of SOD activity. Despite that, SOD activity only shows a slight increasing trend without statistical significance, with the exception for 48 hours and for the highest Cd concentration. Although this data seems contradictory, it is supported by other studies which also refer this fact (87, 88). That is probably due to Cd displacement of Zn ion in cell and in SOD active center, promoting a decrease in its activity (89). Moreover, the data suggests that Cd is inducing H_2O_2 by other sources than SOD. At the same moment Cd also induces oxidative stress by the displacement of Fe, which can produce hydroxyl anion by fenton chemistry (90-92). Moreover, CAT activity increase can also increment the presence of molecular oxygen which in their turn can be another source of free radical (eg. interactions with mitochondria)(13).

Another enzyme that can degrade H_2O_2 is glutathione peroxidase, leading to water as final product. This enzyme uses the reductive power of reduced glutathione (GSH), so is intimately linked to the GSH/GSSG cellular content. GSH is a low molecular thiol that besides being a GPx substract, also may act, alone, as an antioxidant agent, its reported that GSH its responsible by almost 80% of total radical scavenging in cell (93). GSH can be

produced in the cell by *de novo* synthesis, but its majority is recycled by GR from its dimerized form, GSSG. It has been described that Cd has high affinity to GSH and can impair GR activity (11, 30, 93) these results are fully corroborated for the GR activity on MG-63 cell line. GR activity significantly decreased in about 38 and 36% in 50 μM and 20 μM for 24 hours exposure. This significant decrease in GR could result in a significant lowering of total antioxidant capability and in an immediate H_2O_2 increase. In line with GR activity, is total glutathione content the small protein thiol which is the substrate of glutathione peroxidase, it is significantly depleted mainly at 24 hours. This results was been described by other authors in other models (5, 79). This data signifies that Cd is acting in at least 2 fronts, it is boosting ROS production and depleting oxidative defenses, namely, SOD, glutathione cycle, and GSH content.

Our data indicates that Cd also increases cell death by apoptosis. The data from Annexin V assay shown that already at 24 hours Cd induces apoptosis. Cd promoted the decrease in the number of live cells when compared to control, and a significant increase in the cells in early apoptosis (20 μM Cd). Furthermore, data for 48 hours show the same trend. In fact, for this time Cd exposed cells shown an apoptosis profile with both concentration undergoing early apoptotic events and in the case of 50 μM a significant number of cells are in late apoptosis or dead. This may be probably linked to Cd-induced ROS, because it is well known that ROS generation is intimately associated with apoptosis (94, 95)

Altogether, Cd in MG-63 cell line induces apoptosis, which is probably promoted by the oxidative stress imbalance. Because Cd does not produce directly free radicals it must promote oxidative imbalance in an indirect way, increasing redox metal in cytoplasm or disrupting oxidative defenses all of this promote Cd-induced damage.

Moreover, our data show that Cd, even at sub-lethal doses, promotes oxidative stress. This conjugated with the Cd capability to interact with genomic machinery can in long-term result in Cd carcinogenicity as has been reviewed by Valko (23). In fact, the hormesis effect, evidenced at very low concentrations (less than 20 μM), is probably the key to Cd carcinogenicity. In particular, Cd seems to increase ROS which by its turn can promote cellular viability. This increase linked to Cd interaction with genome can in long-term expositions lead to genotoxicity and eventually to cancer.

Conclusions and Future Perspectives

In conclusion, these results suggest that Cd can induce physiological alteration on MG-63 cell line by the disruption of cellular redox state. In particular Cd enhances CAT activity, impairs SOD activity, significantly inhibits GR and lowers the glutathione content. These alterations finally results in cell death.

Based on the results obtained we propose a model for Cd-induced oxidative stress on MG-63 cell line. Cd induces an increase on oxidant species and at the same time inhibits glutathione cycle. Moreover the CAT increase promotes the molecular oxygen increase inside cell. The molecular oxygen can then interact with several molecules or organelles (e.g. mitochondria) creating a loop in which the antioxidant enzyme can promote superoxide formation. The two factors acting synergically, enhances ROS production and lower antioxidant defenses leading to oxidative stress imbalance, and finally to cell death via apoptosis (Figure 9).

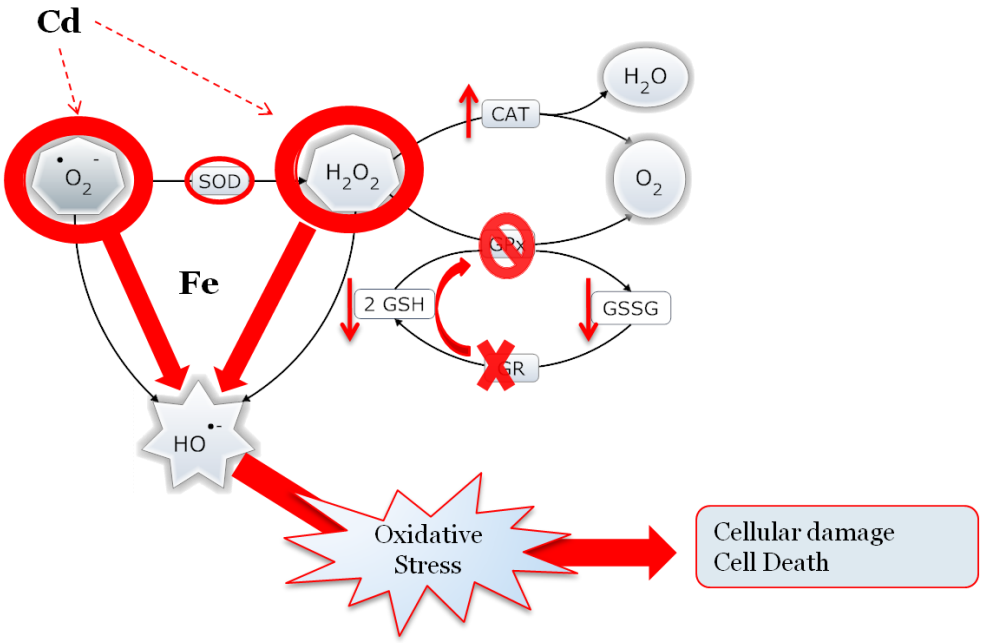

Figure 9 –Proposed model for Cd-induced oxidative stress on MG-63 cell line

To fully understand Cd-induced toxicity and complete the proposed model, future work should be done. Namely, the GPx specific activity is important to corroborate the Cd impairment of the glutathione cycle. Assessment of calcium and iron homeostasis it is important to elucidate primary ROS production, as well as other possible Cd targets. Moreover, quantification of ROS, either H_2O_2, superoxide anion or hydroxyl ion. It is also important damage assessment, like lipid peroxidation and/or protein carbonyl content. Finally, other parameters such as mitochondrial integrity, mitochondrial membrane potential, caspases activation, cytocrome c release, among others, could also clarify the Cd-induced apoptotic pathway.

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