DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

EFFECT OF SHORT-TERM Cr(VI) EXPOSURE ON ENERGY METABOLISM IN HUMAN LUNG EPITHELIAL CELLS

Joana Felício Cerveira

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica da Professora Doutora Ana M. Urbano (Universidade de Coimbra), do Professor Doutor José M. Cuezva (Universidade Autónoma de Madrid) e da Doutora María Sánchez-Aragó (Universidade Autónoma de Madrid)

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DEPARTAMENTO DE BIOLOGÍA MOLECULAR

UNIVERSIDAD AUTÓNOMA DE MADRID

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2011

Declaro que esta dissertação de candidatura ao grau de Mestre é da minha autoria e que os dados aqui incluídos são o resultado de trabalho original por mim efectuado, excepto quando assinalado no texto.

I declare that this thesis, submitted for the degree of Master of Science, is my own composition and that the data presented herein is my own original work, except when stated otherwise.

_____

Joana Felício Cerveira

Agradecimentos

A todas as pessoas que me ensinaram que em qualquer projecto de Ciência não existe eu, mas nós. Agradeço a todas elas que de uma forma ou outra contribuíram para a realização deste trabalho.

À Professora Doutora Ana Urbano por todo o conhecimento que me transmitiu não apenas na realização deste projecto, mas ao longo dos últimos anos. Por todo o acompanhamento, atenção e disponibilidade com que sempre me recebeu. Agradeço também a confiança e amizade que depositou em mim ao longo deste tempo, ensinando-me a procurar ser uma melhor cientista.

Al Profesor Doctor José Cuezva por la gran oportunidad de trabajar en su laboratorio. Le agradezco todo el conocimiento transmitido, las aportaciones y comentarios brindados durante el trabajo. Le agradezco también el haberme facilitado siempre los medios suficientes para llevar a cabo todas las actividades propuestas durante el desarrollo de esta tesis.

A la Doctora María Sánchez-Aragó por haber depositado su confianza en mí, por la paciencia y por el seguimiento de este proyecto. No cabe duda que su participación ha enriquecido el trabajo realizado y, además, no me podría haber tocado mejor "jefita" para esta tesis.

A todo el laboratorio 326 por la acogida y hospitalidad. Os agradezco vuestra total disponibilidad y apoyo, el haber tenido la oportunidad y el placer de compartir el día a día con vosotros. Un agradecimiento especial al "barrio chino" ya que no será lo mismo no trabajar más al ritmo de "Malú".

À unidade Química Física Molecular por me ter dado a oportunidade de realizar parte do trabalho nas suas instalações. Agradeço a simpatia e disponibilidade de toda a equipa e coordenadores para me ajudar. Agradeço também ao pessoal da biblioteca por todos os bons momentos passados na sua companhia.

Aos amigos maravilhosos que fiz em Coimbra… porque Coimbra até podia ser, mas não era a mesma coisa sem vocês. Obrigada por todos os bons momentos que passámos e pelas grandes pessoas que são.

Aos amigos maravilhosos que fiz em Madrid... obrigada pela amizade e companhia nesta aventura em que todos embarcámos.

A toda a minha família. Obrigada por me mostrarem que a união faz a força e ultrapassa todos os obstáculos.

Aos meus pais deixo o agradecimento mais sentido. Agradeço o apoio incondicional que depositaram em mim ao longo de toda a minha vida. Obrigada por serem os melhores pais do mundo e por me terem incutido princípios e valores que fazem de mim o que sou hoje. Obrigada por estarem sempre presentes nos momentos mais importantes. Em particular, obrigada por me proporcionarem esta aventura por Madrid e possibilitarem a realização deste trabalho.

Ao Rodrigo. Obrigada pela compreensão, motivação constante e inspiração em todos os momentos, sejam eles de alegria ou mais difíceis. Sabes o que significas para mim e não teria a mesma piada se o escrevesse. Obrigada por tudo.

Abbreviations and Symbols

$\Delta\psi$	Mitochondrial membrane potential
βF1	β subunit of the mitochondrial H^+-ATP synthase (β-F1-ATPase)
2-DG	2-Deoxiglucose
ADP	Adenosine diphosphate
Asc	Ascorbate
ATP	Adenosine triphosphate
BEAS-2B	Bronchial epithelial airway system 2B
BEC	Bioenergetic cellular
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
COX	Cytochrome *c* oxidase
Cys	Cysteine
DCF	2',7'-Dichlorofluorescein
$DCFH_2$	2',7'-Dichlorodihydrofluorescein
DCFH-DA	2',7'-Dichlorodihydrofluorescein diacetate
DNA	Deoxyribonucleic acid
DNP	2,4-Dinitrophenol
DNPH	2,4-Dinitrophenylhydrazine
DNPhydrazone	2,4-Dinitrophenylhydrazone
ECACC	European Collection of Cell Cultures
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ESR	Electron spin resonance
FACS	Fluorescence-activated cell sorting
$FADH_2$	Flavin adenine dinucleotide (reduced)
FBS	Foetal bovine serum
FL*n*	Optical filter number *n*
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Glut-1	Glucose transporter 1
GSH	Glutathione (reduced)
HIF-1	Hypoxia-inducible transcription factor
HIF-1α and β	Hypoxia-inducible transcription factor subunits α and β
HK	Hexokinase

HK-2	Hexokinase isoform 2
Hsp60	Heat shock protein 60
IARC	International Agency for Research on Cancer
IGF2	Insulin-like growth factor 2
LDH	Lactate dehydrogenase
mtDNA	Mitochondrial DNA
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NAD^+/NADH	Nicotinamide adenine dinucleotide (oxidized/reduced)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NHBE	Normal human bronchial epithelial
OCR	Oxygen consumption rate
OL	Oligomycin
OXPHOS	Oxidative phosphorylation
PBS	Phosphate-buffered saline
PDH	Pyruvate dehydrogenase
PDK-1	Pyruvate dehydrogenase kinase isoform 1
PFK-2	6-Phosphofructo-2-kinase
PHD	Prolyl hydroxylases
PI	Propidium iodide
PPP	Pentose phosphate pathway
PVDF	Polyvinylidene fluoride
Rho^0	Devoid of mitochondrial deoxyribonucleic acid
ROS	Reactive oxygen species
SDH	Succinate dehydrogenase
SDS - PAGE	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SV40	Simian virus 40
TBS-T	Tris buffered saline – tween 20
TCA	Tricarboxylic acid
TEMED	N,N,N',N'-Tetramethylethylenediamine
Trx	Thioredoxin
Trx1 and 2	Thioredoxin isoforms 1 (cytosolic) and 2 (mitochondrial)
VEGF	Vascular endothelial growth factor
VHL	von Hippel-Lindau

Summary

Most of the tumors have a particular energy metabolism strongly relying on glycolysis to fulfil their energetic and biosynthetic demands. This observation was first described by Otto Warburg in the 1920s, who reported that the metabolic switch from cellular respiration to glycolysis takes place even in the presence of oxygen and may be an important event in tumorigenesis. Nowadays, this particular phenotype known as the Warburg phenotype is considered a hallmark of cancer cells.

Chromium (Cr) has been used with industrial and commercial purposes, leading to an environmental and occupational exposure for many humans. The adverse health effects related to Cr-exposure have been identified all over the years and hexavalent chromium [Cr(VI)] is classified as a carcinogenic agent to humans, predisposing for lung cancer. However, in spite of extensive studies, the mechanisms underlying Cr(VI)-induced carcinogenesis remain essentially unknown. In particular, very little is known about the metabolic reprogramming that may be induced by Cr(VI) exposure. To further investigate if the metabolic switch is operating in Cr(VI)-induced carcinogenesis, BEAS-2B cells were exposed to a single and sub-cytotoxic insult of Cr(VI) and its effects in the rates of glycolysis and oxygen consumption, ATP production and in expression levels of the glycolytic marker GAPDH and of mitochondrial activity, the catalytic subunit βF1 from the H^+-ATP synthase were determined. Additionally, we assessed the effects of Cr(VI) exposure in the production of reactive oxygen species (ROS) and carbonylation of proteins. A single exposure to Cr(VI), using a concentration that does not alter the clonogenic potential of cells, decreases the oxygen consumption. This change is paralleled by an increase in the glycolytic flux (higher levels of lactate production). Moreover, Cr(VI)-treated cells display higher levels of the glycolytic marker GAPDH which are correlated with a lower bioenergetic signature (βF1/GAPDH ratio), a metabolic parameter previously found to be altered in many types of carcinomas. No alterations in the production of ATP are observed after a single exposure to Cr(VI). A short-term exposure to Cr(VI) does not induce the production of ROS nor the carbonylation of proteins.

We have demonstrated that human bronchial epithelial cells treated with a single and sub-cytotoxic concentration of Cr(VI) experience a reprogramming of cellular metabolism. This work contributes to our understanding of the metabolic and functional changes underlying Cr(VI)-induced lung carcinogenesis.

Key Words: Lung Cancer, BEAS-2B Cells, Cr(VI) Short-term Exposure, Metabolic Shift

Resumo

A maioria dos tumores apresenta um metabolismo energético glicolítico de modo a obter energia e os requisitos biossintéticos que necessita. Este fénotipo foi inicialmente observado por Otto Warburg nos anos 20, que constatou que esta alteração metabólica da respiração celular para a glicólise ocorre mesmo na presença de quantidades normais de oxigénio, sugerindo que este pode ser um evento importante no processo tumorigénico. Actualmente, este fénotipo particular, conhecido como fenótipo de Warburg, é considerado uma característica basilar das células cancerígenas.

O Crómio (Cr) é amplamente utilizado com fins industriais e comerciais, levando a que muitos humanos sejam expostos a este metal. Os efeitos ao nível da saúde relacionados com a exposição a Cr à muito que foram identificados, sendo o crómio hexavalente [Cr(VI)] classificado como um agente carcinogénico para humanos, predispondo para o aparecimento de cancro do pulmão. Apesar do número extensivo de estudos, os mecanismos celulares e moleculares relacionados com a expoisção a Cr(VI) ainda nao estão totalmente desvendados. Em particular, pouco é sabido sobre a reprogramação metabólica que poderá ocorrer. De modo a investigar se alterações metabólicas ocorrem durante a carcinogénese induzida por Cr(VI), células BEAS-2B foram expostas a um único insulto sub-citotóxico de Cr(VI) e os seus efeitos ao nível do fluxo glicolítico, produção de ATP e níveis de expressão dos marcadores de glicólise, GAPDH, e de actividade mitocondrial, subunidade catalítica βF1 da H^+-ATP sintase, foram determinados. Adicionalmente, testámos os efeitos da exposição a Cr(VI) na produção de espécies reactivas de oxigénio e na carbonilação de proteínas celulares. Uma exposição única a Cr(VI), usando uma concentração que não afecta o potencial clonogénico das células, leva a uma diminuição do consumo de oxigénio. Esta alteração ocorre paralelamente com o aumento do fluxo glicolítico (maiores níveis de produção de lactato). Além disso, células tratadas com Cr(VI) têm um maior nível de expressão do marcador glicolítico, o que se correlaciona com uma menor assinatura bioenergética (razão βF1/GAPDH). Não são observadas alterações na produção de ATP após tratamento único com Cr(VI). Um único insulto de Cr(VI) não induz a produção de espécies reactivas de oxigénio nem a produção de proteinas celulares carboniladas.

Com este trabalho demonstrámos que células epiteliais humanas de pulmão expostas a um único tratamento sub-citotóxico com Cr(VI), sofrem uma reprogramação do seu metabolismo energético, o que contribui para o nosso conhecimento das alterações metabólicas e funcionais que ocorrem no desenvolvimento de cancro de pulmão.

Palavras-chave: Cancro do Pulmão, Células BEAS-2B, Exposição curta a Cr(VI), Alteração Metabólica

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Chapter 1
INTRODUCTION

1.1) The Metabolism of Glucose in Mammalian Cells

In most normal mammalian cells glucose is the preferred metabolic substrate for the generation of energy and building blocks required for maintenance, accretion and/or proliferation. The extraction of energy from glucose can occur by two general processes: glycolysis (with lactate production) and cellular aerobic respiration (Fig. 1.1), the latter comprising three major pathways: glycolysis, tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS) - and allowing the complete oxidation of glucose. These pathways take place in the cytosol (glycolysis) and in mitochondria (TCA cycle and OXPHOS). In anaerobic glycolysis, glucose is not completely oxidized and lactate is formed (see below) [1,2].

During glycolysis (Fig. 1.1), a molecule of glucose is partially oxidized yielding two molecules of pyruvate. Some of the free energy released from the hexose is conserved in two molecules of adenosine triphosphate (ATP) and two molecules of reduced nicotinamide adenine dinucleotide (NADH) [1,2]. The glycolytic flux is strictly regulated by several mechanisms in order to maintain constant the levels of ATP. These mechanisms involve a complex interaction between ATP consumption, NADH regeneration, allosteric regulation of several glycolytic enzymes and the levels of key metabolites. Among the glycolytic enzymes, some such as hexokinase (HK, EC 2.7.1.1), 6-phosphofructo-1-kinase (EC 2.7.1.11) and pyruvate kinase (EC 2.7.1.40) are strictly regulated. The key metabolites for the regulation of glycolysis are fructose 2,6-bisphosphate and cyclic adenosine monophosphate (cAMP) that reflects the cellular balance between ATP production and consumption. Moreover, glycolysis is also regulated by the hormones glucagon, epinephrine and insulin, and by changes in the expression levels of the genes encoding several glycolytic enzymes [2].

When cells have a limited supply of oxygen, glycolytic pyruvate is reduced to lactate promoting the regeneration of oxidized nicotinamide adenine dinucleotide (NAD^+) from NADH. This reaction is catalyzed by lactate dehydrogenase (LDH, EC 1.1.1.27) and the excess of lactate is excreted by the cell [1,2].

However, under aerobic conditions mammalian cells oxidize pyruvate to CO_2 by the sequential activity of pyruvate dehydrogenase (PDH, EC 1.2.4.1) and of the TCA cycle, forming ATP and the reduced coenzymes NADH and flavin adenine dinucleotide ($FADH_2$) [1,2]. In the mitochondrial inner membrane, there is a chain of electron-carrier compounds, known as respiratory chain that drives electrons from the reduced coenzymes to molecular oxygen, which acts as the terminal electron acceptor. The energy of the electron transfer is efficiently

conserved in a proton gradient, resulting from the proton pumping from the mitochondrial matrix to the inter-membrane space through the proteic complexes. The electron-carrier chain is constituted by four (I to IV) protein complexes. For each pair of electrons transferred to O_2 from NADH, four protons are pumped out of the mitochondrial matrix by complex I, four by complex III and two by complex IV [1,2]. With $FADH_2$ as the electron donor, the electron-carrier complexes pump a total of six protons. The electrochemical energy inherent to this difference in proton concentration and separation of charge represents a temporary conservation of much of the energy of electron transfer. The energy stored in such a gradient is termed the proton-motive force. The re-entry of protons into the mitochondrial matrix through the H^+-channel of the mitochondrial H^+-ATP synthase (EC 3.6.1.34) drives the synthesis of ATP in OXPHOS [1, 2]. The terminal oxidation of pyruvate in mitochondria yields almost 20-fold more ATP than glycolysis. On the other hand, glycolysis can occur in the absence of oxygen, being a quick process that occurs a hundred times faster than cellular respiration [1-3].

The use of glucose by the cell is not restricted to its oxidation for ATP generation. Another possible fate of glucose is to be catabolised in the pentose phosphate pathway (PPP) to obtain reducing power in the form of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and biosynthetic intermediates that can be used in different anabolic processes (Fig. 1.1), such as lipogenesis. Noteworthy, ribose 5-phosphate necessary for nucleic acid synthesis, and NADPH, the principal reducing power in many biosynthetic reactions, are both provided by the PPP. Even when glucose is converted to pyruvate and this molecule is partially oxidized in the TCA cycle, some of the TCA intermediates could be used for biosynthetic purposes. For instance, oxaloacetate and α-ketoglutarate, both intermediates of TCA cycle, are precursors of aspartate and glutamate, respectively, which can be used for synthesis of other amino acids, purines and pyrimidines. Citrate is a precursor of fatty acids and steroids. The usage of TCA cycle intermediates for biosynthetic purposes drives anaplerotic reactions to re-establish their levels and ensure the maintenance of the correct flux. Glutaminolysis provides oxaloacetate through glutamine catabolism. Also, the conversion of glutamine to lactate generates reducing power in the form of NADPH.

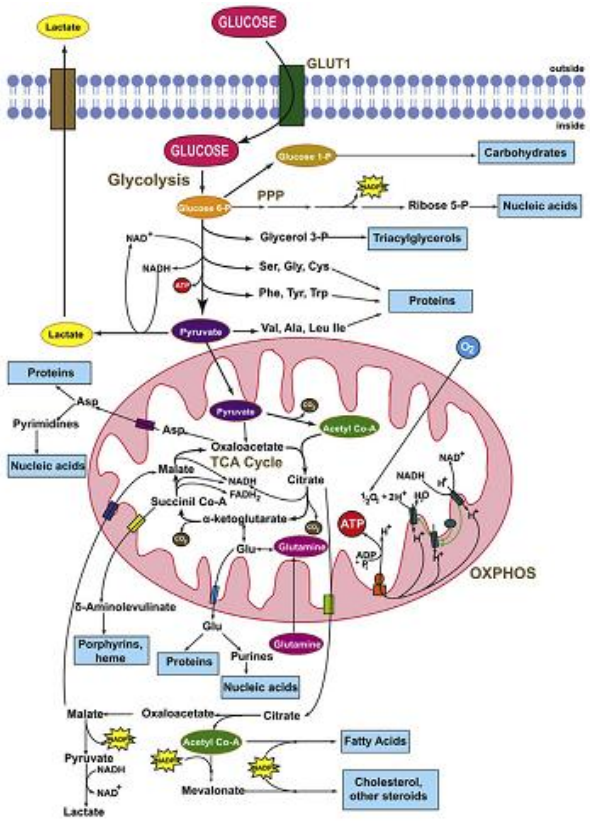

Fig. 1.1- Pathways of glucose metabolism. The model shows some of the relevant aspects of the metabolism of glucose. After entering the cell by specific transporters, glucose is partially oxidized in glycolysis to generate energy, reduced power and pyruvate. In the cytosol, the generated pyruvate can be reduced to lactate and excreted by the cell or oxidized in the mitochondria by pyruvate dehydrogenase to generate acetyl CoA, which enters in the TCA cycle. The operation of the TCA cycle completes the oxidation of acetyl CoA. The transfer of electrons obtained in biological oxidations (transported by NADH/$FADH_2$) to molecular oxygen by respiratory complexes of the inner mitochondrial membrane (in green) is depicted by yellow lines. The utilization of the proton gradient generated by respiration for the synthesis of ATP by the ATP synthase (in orange) in OXPHOS is also indicated. Glucose can also be catabolized by the PPP to obtain reducing power in the form of NADPH, be used for the synthesis of other carbohydrates or utilized to generate other metabolic intermediates that could be used in different anabolic processes (blue boxes). Different pathways that drain intermediates of the TCA cycle (oxaloacetate, succinil-CoA, α-ketoglutarate and citrate) for biosynthetic purposes are represented (blue boxes). Abbreviations: PPP, pentose phosphate pathway; NADPH, reduced nicotinamide adenine dinucleotide phosphate; acetyl CoA, acetyl coenzyme A; TCA, tricarboxylic acid; NADH/NAD^+ reduced/oxidized nicotinamide adenine dinucleotide; ATP, adenosine triphosphate; $FADH_2$/FAD, reduced/oxidized flavin adenine dinucleotide; OXPHOS, oxidative phosphorylation [4].

1.1.1) Role of Mitochondria in Cellular Physiology

Mitochondria are membrane-enclosed organelles, a few micrometers long, found in most eukaryotic cells. The number of mitochondria in a cell varies widely by organism and tissue type. This organelle is compartmentalized, and specialized functions occur in its different regions. These compartments or regions include the outer membrane, the intermembrane space, the inner membrane and the matrix. The outer membrane has a high

permeability to small molecules and ions, whereas the inner membrane is practically impermeable to all polar molecules and ions, including protons. The inner mitochondrial membrane includes, among other components, the respiratory electron carriers (complexes I to IV), the adenine nucleotide translocase and the H^+-ATP synthase (complex V). The mitochondrial matrix contains the PDH complex and the enzymes of the TCA cycle, amino acid oxidation and β-oxidation pathways. In the matrix is also found deoxyribonucleic acid (DNA), ribosomes, few ions (Mg^{2+}, Ca^{2+}, K^+), and many other enzymes and metabolic intermediates [2].

Some of the protein constituents of the respiratory chain are codified by DNA contained in mitochondria (mtDNA). However, only 13 polypeptides necessary for mitochondria are actually coded by mtDNA and the greater part of them are nuclear-encoded. Though, all OXPHOS complexes, with the exception of complex II, contain at least one polypeptide codified by the mtDNA. [5,6].

The H^+-ATP synthase is an F-type ATPase that contains two different subcomplexes. The F1-ATPase sub-complex contains the subunit β-F1-ATPase (βF1), which protrudes into the matrix of the mitochondria and has the catalytic activity necessary for the synthesis of ATP. The Fo sub-complex, which is integral to the membrane and acts as a proton channel, allows the re-entry of H^+ to the mitochondria matrix for the synthesis of ATP [2].

The function of mitochondria in the cell is not limited to the production of ATP. They play essential roles in the execution of cell death and in intracellular signalling of calcium and reactive oxygen species (ROS). Importantly, these different processes are interconnected. The production of ROS and the activation of the mitochondrial-dependent cell death pathway are linked to the energy metabolism. The maintenance of the mitochondrial membrane potential ($\Delta\psi_m$) within certain limits is very important, because when $\Delta\psi_m$ increases the formation of ROS augments exponentially [7]. In non-glycolytic cells treated with an apoptosis inducer, H^+-ATP synthase activity supports high $\Delta\psi_m$ and the subsequent generation of ROS. The formation of ROS, controlled by the activity of H^+-ATP synthase, is a death signal required for efficient execution of programmed cell death. Apoptosis is initiated by release of ROS-modified apoptogenic molecules from mitochondria [8].

Therefore, a growing number of human diseases are now associated with molecular and/or functional alterations in mitochondria.

1.2) Cancer, Metabolism and Mitochondria

Cancer is considered a complex genetic disease that confers to cells three general properties: unlimited replicative potential, metastasis and colonization of different tissues. The transformation of normal cells into progressive malignancy is a multistep and sequentially ordered process. In addition to the contribution of genetic mutations in cancer genes, the progression of cancer is also bound to the cancer cell environment and to other epigenetic events [5].

In this regard, Hanahan and Weinberg in 2000 identified six general traits of the cancer cell: self-sufficiency in growth signals, insensitivity to anti-growth signals, limitless replicative potential, evasion of apoptosis, sustained angiogenesis and tissue invasion and metastasis [9]. In the ensuing decade, carcinogenicity studies showed that the carcinogenic process is much more complex and involves modifications in other cellular properties. As a consequence, Hanahan and Weineberg, in 2011, have proposed the addition of two emerging hallmarks (Fig. 1.2), namely the deregulation of cellular energetics and the avoidance of immune destruction. They have also proposed two cellular modifications that enable the acquisition of these eight hallmarks during carcinogenesis: cells with altered genotypes will have a selective advantage to grow in a specific tissue environment and virtually all neoplasias are infiltrated in different degrees with immune system cells [10] (Fig. 1.2).

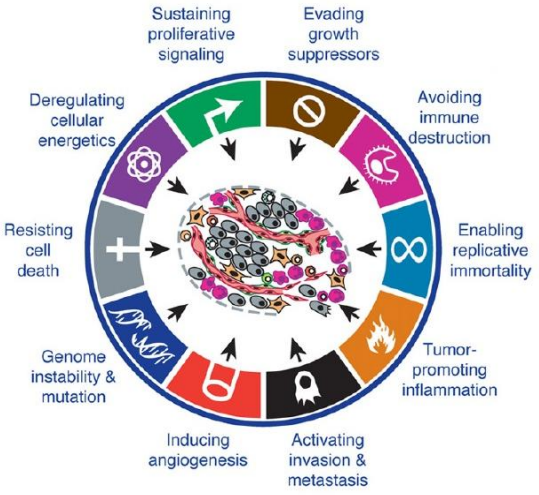

Fig. 1.2- The hallmarks and enabling characteristics of cancer cells. The properties identified by Hanahan and Weinberg altered in cancer cells are schematically represented. They proposed the existence of eight modifications acquired during the process of tumorigenesis by cells. Underlying the hallmarks are two enabling characteristics, genome instability and tumor-promoting inflammation. Adapted from [10].

1.2.1) An Emerging Hallmark: Metabolic Reprogramming

One of the recently proposed emerging hallmark of cancer, the so called "metabolic reprogramming" [11,12] was, in fact, partially described by Otto Warburg almost a century ago. The German scientist observed that tumors have an abnormally high aerobic glycolysis and suggested that the bioenergetic activity of mitochondria was impaired in the cancer cell [13,14].

In the 1920s, Otto Warburg first reported that, even in the presence of oxygen, tumor cells consume larger amounts of glucose and produce higher amounts of lactate than their normal counterparts [13,14]. The phenomenon became known as the Warburg effect.

In his extensive metabolic experiments, Warburg measured the amount of oxygen consumed by tumor slices and mouse ascites cancer cells (which he considered almost pure cultures of cancer cells) and correlated the values obtained to the quantity of ATP produced by cellular respiration or by glycolysis. Surprisingly, he found that cancer cells obtained almost the same amount of energy from glycolysis as from respiration, whereas non-malignant cells produced most of their energy by respiration. Warburg ascribed this observation to an impairment of the bioenergetic function of mitochondria in cancer cells, suggesting that this metabolic change is the determinant event in tumorigenesis [13].

Warburg's hypothesis was considered by many authors as a simple Pasteur Effect, which settled that oxygen availability controls the rate of glucose utilization by the cells [15-17]. Warburg's postulates were debated for many years, and finally considered an irrelevant epiphenomena of cell transformation, until their recent rediscovery mostly propitiated by the use of 2-[^{18}f]fluoro-2-deoxyglucose - positron emission tomography imaging in oncology [18].

1.2.2) Mechanisms Underlying the Warburg Effect

Cancer cells demand higher levels of energy and biosynthetic precursors, due to their fast proliferating characteristics. Their stronger reliance on glycolysis to fulfil these demands causes them to consume more glucose and produce more lactate [19]. Diverse mechanisms have been proposed to explain the metabolic switch from mitochondrial respiration to aerobic glycolysis experienced by tumor cells.

Due to higher rates of proliferation, a hypoxic microenvironment can be created within cancer cells. Hypoxia results in the stabilization of the hypoxia-inducible transcription factor (HIF-1). HIF-1 is constituted by two subunits: HIF-1β, expressed constitutively, and HIF-1α, whose levels are dependent on the oxygen pressure in tissues and/or on the activation of specific growth factors. Thus, the formation of HIF-1 complex is mainly regulated by HIF-1α

levels [3,20,21]. Under normoxia, the α-subunit is rapidly degraded by the ubiquitin-proteasome system. The degradation of HIF-1α depends on the activation of HIF-prolyl hydroxylases (PHD, EC 1.14.11.2) and the von Hippel-Lindau (VHL) protein that allows the degradation by 26S-proteasome [3,20,21]. The lack of VHL allows the stabilization of HIF-1α even in normoxia, because the proteasome-dependent degradation is blocked [22]. The stabilization of HIF-1α induces the expression of the glucose transporter 1 (GLUT-1) and many glycolytic enzymes, such as aldolase (EC 4.1.2.13) and enolase (EC 4.2.1.11), LDH and the glycolytic regulator 6-phosphofructo-2-kinase (PFK-2)/fructose-2,6-biphosphatase, as well as the factors that trigger angiogenesis (vascular endothelial growth factor, VEGF), cell growth and survival pathways (insulin-like growth factor 2, IGF2) [23-25]. HIF-1 also regulates the activity of complex IV by stimulating the expression of cytochrome *c* oxidase (COX) subunit 2 (COX4-2), instead of COX4-1. This variation of isoforms leads to an optimization of the efficiency of OXPHOS by increasing complex IV activity, when O_2 is a limiting factor. The higher efficiency of COX4-2 enables an attenuation of ROS production [26]. It has also been suggested that the glycolytic phenotype can be promoted through cooperation of HIF-1α with the oncogene c-Myc (when this one is over-expressed) by induction of pyruvate dehydrogenase kinase isoform 1 (PDK-1), hexokinase isoform 2 (HK-2) and LDHA [27]. PDK-1 phosphorylates and inactivates the PDH complex, inhibiting the mitochondrial metabolism of pyruvate. Frequently, tumors overproduce HK-2, which neutralizes the capacity of glucose 6-phosphate to inhibit glycolysis [28]. The high levels of glucose 6-phosphate can also stimulate the PPP that provides intermediates for biosynthesis and leads to increased levels of the coenzyme NADPH. Importantly, the formation of NADPH leads to an accumulation of reduced glutathione (GSH) that acts as a ROS scavenger, thus supporting cell survival [28-31]. In renal carcinoma cells lacking the VHL protein, the biogenesis of mitochondria can be indirectly repressed by gain-of-function of HIF-1α. HIF-1 mediates this effect again by interacting with c-Myc [32]. Overall, the activation of HIF-1 complex results in the stimulation of the glycolytic flux and attenuation in the mitochondrial activity [27].

Oncogenes *per se* can also activate glycolysis. Activation of the Akt and Ras oncogenes results in increased glucose transport and stimulation of HK-2 and PFK-2 activity, which enhances glycolytic rates [3,33]. There are evidences that Myc oncogene activates glycolytic genes and mitochondrial biogenesis, which when sustained by high Myc levels can result in ROS production [3,33]. The oncogene Akt seems to promote cell survival by a glucose hydrolysis-dependent mechanism after growth factor withdrawal [34]. In addition, Akt stimulates the oxidation of glucose, which correlates with tumor aggressiveness *in vivo* [35]. In

non-invasive radial growth melanomas, the over-expression of Akt induces the expression of glycolytic markers, as well as a shift to malignant and invasive vertical-growth phenotype [36]. The increased glucose uptake mediated by Akt stimulates the formation of NADPH in PPP, which is necessary for *de novo* lipogenesis [37].

The tumor suppressor protein p53 is also important in promoting the metabolic shift from mitochondrial processes to glycolysis. Besides its role in cell cycle control, p53 has an important function in the control of the metabolic status of the cell. In normal conditions, p53 leads to stimulation of cellular respiration, because it stimulates the expression of COX2 protein that is necessary for the assembly of COX complex [38]. Moreover, p53 acts as a negative regulator of glycolysis under glucose deprivation, because it can induce cell cycle arrest until fuel restoration [39]. In several types of cancer cells, p53 functions are more or less lost, causing the suppression of OXPHOS and glycolysis up-regulation [39, 40].

Mutations in mitochondrial genes codified by mtDNA are also associated with tumorigenesis. These mutations can diminish complexes I and III activities and increase ROS production [41]. mtDNA has characteristics that make it an easy target for genetic damage, like the location nearby ROS generation sites, lack of protection by histones and a weak capacity of DNA repair in the mitochondria. In fact, mtDNA mutations have been observed in leukemia, prostate, gastric and breast cancers [42]. Experiments with cells devoid of mtDNA (rho^0 cells) provide evidences that mtDNA can be important in the process of malignant transformation. Rho^0 cells derived from breast tumors and glioblastomas lost some of their tumorigenic characteristics when the mtDNA was removed and the same malignant features were restored upon introduction of normal DNA-containing mitochondria. Rho^0 HeLa cells cannot form tumors when injected into nude mice, but this capability is restored upon introduction of mtDNA from normal human fibroblasts [43]. Moreover, mutations in nuclear-encoded mitochondrial genes are also found in some types of cancers. Mutations in the codifying genes of succinate dehydrogenase (SDH, EC 1.3.5.1), a TCA cycle enzyme, cause the accumulation of succinate, which in turn inhibits PHD and HIF-1α degradation. Inherited or somatic mutations in SDH codifying genes are associated with the development of phaeochromocytoma and paraganglioma [44,45].

The over-expression and/or downregulation of the above-mentioned genes cannot be the only events responsible for the development and sustenance of a glycolytic phenotype, since the metabolic reprogramming is found in the most prevalent types of cancers and the frequency of mutations in these genes is lower [4]. So, the progression of malignancy may also be triggered by non-mutational events. In this regard, plausible modifications that may be

linked to the metabolic reprogramming are the structural and functional impairments of mitochondria, since many metabolic and bioenergetic functions take place in this organelle [4,46].

Indeed, a proteomic approach used to determine the expression of both glycolytic and mitochondrial proteins has revealed an altered pattern in tumors. This approach allows protein estimation of both bioenergetic competence of the organelle (as assessed by the ratio between two mitochondrial proteins, βF1 and heat shock protein 60 (Hsp60)) and overall mitochondrial potential of the cell (as assessed by the ratio between βF1 and the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH)). The latter ratio is defined as the Bioenergetic Cellular (BEC) Index or bioenergetic signature of the cell [46,47]. The analysis of these two ratios in tumors when compared with normal tissues gives information about possible interferences with mitochondrial activities of the cell [4,47]. Several studies have demonstrated a reduction of the bioenergetic competence of mitochondria and an up-regulation of glycolytic flux in many human carcinomas, such as breast adenocarcinomas, gastric adenocarcinomas and squamous carcinomas of the lung [12,48,49]. The bioenergetic signature inversely correlates with the increase of the glycolytic flux, suggesting a relationship between these two parameters in cancer cells [12]. Moreover, the bioenergetic signature could be used as a marker for progression of different types of cancer [47,50,51].

1.3) Chromium

For many years, chromium (Cr) has been used with industrial and commercial purposes, leading to an environmental and occupational exposure for many humans. The adverse health effects related to Cr exposure have also been identified all over these years, especially at the level of the skin and respiratory tract. In particular, the multiple epidemiological studies and the results from *in vitro* and *in vivo* experiments led the International Agency for Research on Cancer (IARC) to classify hexavalent chromium [Cr(VI)] compounds, as encountered in the diverse Cr-related industries, as a carcinogenic agent to humans (group 1) [52]. The collected data revealed a consistent association between exposure to Cr(VI)-containing compounds and an increased risk for lung cancer. However, despite many extensive studies, the mechanisms underlying Cr(VI)-induced carcinogenesis remain essentially unknown. Therefore, the effects exerted by Cr(VI) at the intracellular level, must be dissected in order to better understand the complexity of Cr(VI)-induced carcinogenesis.

1.3.1) Chemical Properties

Cr is a chemical element that belongs to group 6 and to the 4th period of the Periodic Table and has the configuration [Ar]3d^{5}4s^1. Although the oxidation states of chromium range from -II to +VI [53], the only valence states that occur naturally in the environment are the trivalent [Cr(III)] and the hexavalent [Cr(VI)] states, being the latter primarily produced by anthropogenic sources [54,55]. Cr(III) complexes are characterized by a low spin, octahedral geometry. The majority of Cr(III) complexes are kinetically inert [56]. Cr(VI) is a powerful oxidizing agent. The two forms present in aqueous solution are chromate (CrO_4^{2-}) and dichromate (CrO_7^{2-}). Therefore, Cr(VI) exists always as an oxyanion in aqueous solution [55]. Both chromate and dichromate have a tetrahedral structure, but they have different reactivities, being the latter a stronger oxidizing agent [57].

1.3.2) Industrial Applications

Cr is widely used in metallurgical, refractory and chemical industries. In the metallurgical industry, Cr is mostly used to produce ferrous and non-ferrous alloys and stainless steel materials [53]. The stainless steel industry uses a mixture of different metals to produce a component used during the welding processes. These materials contain up to 30% of Cr, in both the trivalent and hexavalent states [58]. In the refractory industry, Cr is used to produce heat-resistant materials, such as firebricks for fumaces, since Cr has a high melting point and resists to acid and alkali damage [53]. In the chemical industries, both Cr(III) and Cr(VI) are used mainly as pigments. For instance, the two forms of Cr(VI) in solution are colourful, being yellow (chromate) or orange (dichromate). Other uses for Cr compounds include Cr(VI) in metal finishing, Cr(III) in leather tanning, and Cr(VI) in wood preservatives [59].

1.3.3) Exposure to Cr and its Physiological Relevance

Cr can be found in air, soil and water due to the manufacture and disposal of chromium-based products. Also, Cr can be found on tobacco smoke. The concentration of Cr in rural and urban areas is generally low (<10 ng/m^3 in rural areas and 0-30 ng/m^3 in urban areas) but, for instance, as a result of smoking the concentration of indoor air contaminated with Cr can be 10-400 times higher [59].

Cr has been identified in the tissues of occupationally-exposed humans and the principal route for the absorption of Cr seems to be the lungs, which depends on the oxidation state, size and solubility of the Cr particles [60,61].

Trivalent compounds normally have low toxicity, being poorly absorbed by the lungs and

gastrointestinal tract [53,54]. In fact, Cr(III) seems to have an important role in insulin metabolism of patients with diabetes, being used as a nutritional supplement [62].

Cr(VI) particles are retained in lung tissue, specially at bronchial bifurcations, where they can persist for twenty years and cause airway hypersensitivity, respiratory distress, fibroproliferative disease and, ultimately, pulmonary carcinogenesis [63]. The Cr(VI) particles that pose the major oncogenic risk are the ones raging from 0.2 to 10 μm, resulting from industrial activities [64]. Fortunately, Cr(VI) present in these particles can be inactivated by mammalian defence mechanisms. This inactivation is done extracellularly by reduction of Cr(VI) to Cr(III). In particular, De Flora *et al* (1997) have demonstrated that whole blood, lung epithelial fluid, alveolar macrophages, and peripheral parenchyma cells have the ability to reduce Cr(VI) to Cr(III) [65]. Systemic Cr(III) does not appear to be stored for extended periods of time within the body tissues [59].

The potential carcinogenic effect of Cr(VI) enters in discussion when it is actively transported to the cells before being reduced. At physiological pH, the predominant form of Cr(VI) is the chromate oxyanion with a tetrahedral structure similar to those of sulfate and the phosphate oxyanions. Therefore, it is non-specifically transported and accumulated by cells [66,67]. On the contrary, Cr(III) forms low-spin, octahedral coordination complexes and chelates, which are very large compounds that cannot easily be transported through the cell membrane [63]. This structural aspect may be one of the causes for the fact that no adverse effects were observed in workers exposed to Cr(III) compounds for up to 25 years, whilst chronic exposure to Cr(VI)-containing compounds was shown to be associated with an increased risk for lung cancer [18-21]. Another possible factor for the greater toxic potency of Cr(VI) relative to Cr(III) is the higher redox potential of Cr(VI). Cr(VI) is rapidly reduced to Cr(III), with Cr(V) and Cr(IV) species as intermediates [68]. These intermediates, as well as Cr(VI) can be involved in the oxidative damage of many cellular constituents (discussed in the subsequent sections).

The most prevalent type of lung cancer reported among Cr(VI) workers is squamous cell carcinoma, a sub-type of non-small cell lung cancer [63,69,70]. Nonetheless, small cell carcinoma was reported as the type usually detected in Slovakia [71].

1.3.4) Intracellular Metabolism of Cr(VI)

In the hexavalent state, Cr has little relevant biological activity, i.e., has a reduced interaction with macromolecules [54]. Inside the cell, and at different sites (cytosol, mitochondria, nucleus and endoplasmatic reticulum), the hexavalent ion is readily reduced to

Cr(III). The non-enzymatic reduction is performed mainly by ascorbate (Asc), GSH and cysteine (Cys) [54]. Of these, Asc seems to be the primary reducer agent, since it has a high intracellular concentration (in the milimollar range) and has a faster reduction kinetics, when compared with GSH or Cys [72]. Other cellular constituents can potentially reduce Cr(VI) to Cr(III), such as hydrogen peroxide, diols, α-hydroxycarboxylic acids, among others [63].

Intracellularly, there are two mechanisms for Cr(VI) reduction. The first one occurs at relatively low levels of Asc, where a series of one electron reduction reactions take place, producing the intermediates Cr(V) and Cr(IV) and, ultimately, Cr(III) (equation 1.1). When the amount of intracellular reductants is not limiting, Cr(VI) is directly reduced to Cr(IV) followed by a one electron reduction to Cr(III) [73, 74] (equation 1.2).

$$Cr^{6+} \xrightarrow{e^-} Cr^{5+} \xrightarrow{e^-} Cr^{4+} \xrightarrow{e^-} Cr^{3+} \quad (1.1)$$

$$Cr^{6+} \xrightarrow{2e^-} Cr^{4+} \xrightarrow{e^-} Cr^{3+} \quad (1.2)$$

GSH and Cys can also reduce Cr(VI) by both pathways [54]. Moreover, in the case of Cys, kinetic studies at neutral pH showed that major route to reduce Cr(VI) is the one-electron transfer [75]. *In vivo*, the formation of one or two Cr intermediates depends not only on the proportion reducer:Cr(VI), but also on additional specifications, like the presence of other oxidants and catalytic metals such as Fe [76-78].

1.3.5) Proposed Mechanisms for Cr(VI) Intracellular Toxicity

The carcinogenic potential of Cr(VI)-containing compounds is very complex and many mechanisms have been proposed. Taking into account that cancer is generally viewed as a genetic disease, many researchers conducted their studies in order to clarify the genotoxic effects of Cr compounds. Cr(VI) does not interact or bind directly with nucleic acids, but its reduction intermediates, and ultimately Cr(III) have a high affinity for DNA, as well as for proteins. [54].

Cr(III) has six coordination sites and, consequently, can form several types of complexes with biomolecules. Ternary DNA-crosslinks with other molecules (GSH, Cys, histidine and Asc) represent the major Cr-DNA adducts present in mammalian cells [79]. In addition, other types of structures can be formed: Cr(III)-DNA binary complexes, DNA-protein crosslinks and DNA inter/intrastrand crosslinks. Single- and double-strand breaks and abasic sites are also observed upon Cr(VI) exposure [54,63]. However, not all adducts appear to have the same mutagenic and, concomitant carcinogenic potential. Under physiological conditions, the Asc-

Cr(III)-DNA cross-links seem to be the most mutagenic lesions (Fig. 1.3) [79].

It has also been proposed that oxidative damage has a major role in Cr-induced toxicity. Several theories proposed that this toxicity is achieved trough Cr intermediate oxidation states (Cr(V) and Cr(VI)). The damage can be induced by direct electron abstraction by Cr species [77], formation of ROS [76] or via Cr(V)-peroxo-intermediate formation [78]. Several studies have shown the participation of Cr(VI), Cr(V) and Cr(IV) in Fenton-like reactions (equation 1.3), producing hydroxyl radicals, in which Cr(V) is continuously recycled into Cr(VI) [80].

$$Cr(V) + H_2O_2 \rightarrow Cr(VI) + {}^{\bullet}OH + OH^{-} \qquad (1.3)$$

The formation of these radicals may be responsible for harmful effects within the cells, namely lipid peroxidation [81], modification of signalling pathways [82, 83] and intracellular constituents, like the cytoskeleton [84,85] and DNA [86]. Some studies performed in animals (rabbits and rodents), associate the formation of ROS induced by Cr(VI) exposure, with liver, testes, brain, kidney and lung injuries [87] and with activation of proteins of the matrix, important to cell invasion and metastasis [88]. Still, some authors defend that the formation of Cr-DNA adducts are the major lesions associated with the carcinogenity of Cr(VI) [79].

The structural and functional damages induced by Cr(VI) on DNA can lead to growth arrest [89] and apoptosis [85,90]. The extent of the damage determines which cellular response is triggered. Also, Cr(VI) exposure can induce dysfunctional DNA replication, transcription and repair [63]. Studies with human lung cells also suggest that chromosome instability is associated with Cr(VI)-induced lung cancers, and the instability appears to be mediated through centrosome and spindle assembly checkpoint bypass [91].

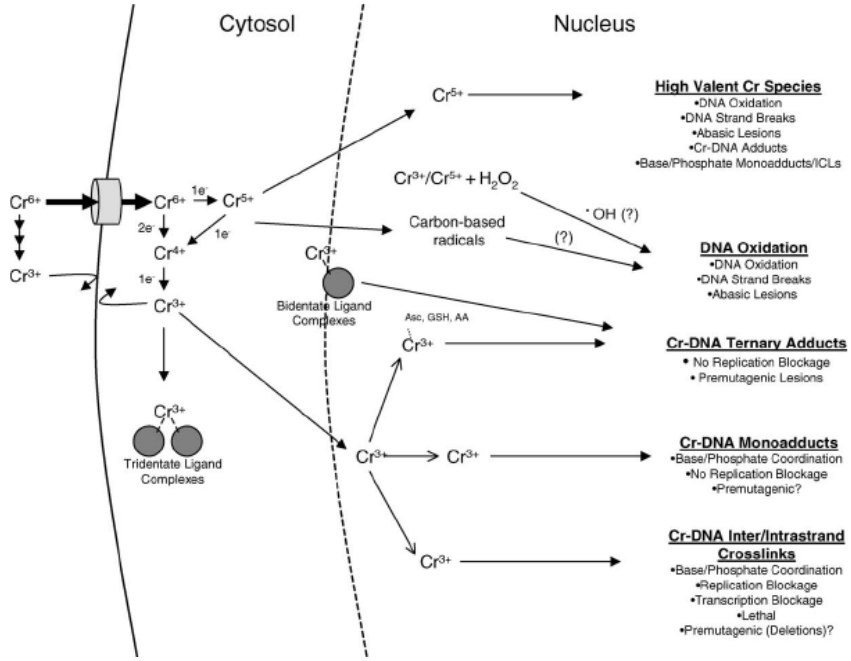

Fig. 1.3- General pathways involved in the genotoxicity induced by Cr. The figure illustrates the relationship between Cr metabolism and DNA damage. Cr^{6+} enters the cell via sulphate/phosphate transporters and is rapidly reduced by a one or two electron mechanism to Cr^{3+}, which is unable to cross the cell membrane. An initial one-electron reduction can lead to the generation of high valent Cr species (Cr^{5+}/Cr^{4+}). Both Cr^{3+} and Cr^{5+} display an appreciable affinity for both DNA bases and the phosphate backbone, leading to the formation of Cr - DNA monoadducts, ICLs and ternary adducts. Cr^{5+} can directly oxidize DNA bases (guanine) and sugars (hydrogen abstraction) and produce DNA strand breaks. Cr^{3+} is the ultimate DNA reactive species and is critical for the formation of monoadducts, ICLs and DNA-protein crosslinks. Both Cr^{3+} and Cr^{5+} may generate the hydroxyl radical in the presence of H_2O_2 and lead to oxidative DNA damage. Abbreviations: ICLs, interstrand crooslinks; H_2O_2, hydrogen peroxide [54].

To better understand the type of response obtained after exposure to Cr(VI), an excellent tool is the study of the signalling pathways affected by this metal. Studies of gene expression profiles have yielded many important data. Nevertheless, this data should be carefully analysed because exposure regimens and the type of cell line used exert different effects on the pattern of gene expression. The importance of adequate concentrations and study models used will be discussed in more detailed in section 1.3.7.

1.3.6) Cr(VI)-Induced Changes of Energy Metabolism

As mentioned before, most cancer cells exhibit a particular metabolism, and this situation should also be a relevant point to dissect when considering Cr(VI)-induced carcinogenity. Noteworthy, few studies have analysed the modifications on bioenergetic parameters after Cr(VI) exposure. The firsts metabolic studies performed have shown that Cr(VI) induces changes in oxygen consumption rate (OCR) and in the adenylate pool [92-95]. These studies were performed either in cultured cells [92,95,96] or in isolated mitochondria

[93,94]. A diminution of ATP levels concomitant with an increase of adenosine diphosphate (ADP) and AMP pools and alterations on the activity of complex I were observed [92-94].

Moreover, electron spin resonance (ESR) studies showed that Cr(VI) inhibits electron flow through complexes I and II, an effect mediated by oxidation of the Fe-S centers present in these complexes [97]. Studies from the same group demonstrated that Cr(VI) interferes with the thioredoxin (Trx) system. This system maintains the intracellular thiol redox balance, promoting cell survival. Treatments with Cr(VI) promote oxidation of both cytosolic and mitochondrial thioredoxins isoforms, Trx1 and Trx2, respectively, interfering with the redox balance of the cell [98,99].

Studies with PC-12 cells showed that exposure to Cr(VI) at sub-cytotoxic concentration resulted in higher glucose uptake and lower adenylate energy charge, supporting a metabolic shift from OXPHOS to aerobic glycolysis [96].

1.3.7) The Influence of the Experimental Conditions in the Study of Cr(VI)-Induced Carcinogenity

Cr(VI) is a well-recognized human lung carcinogen [52]. Extensive information about Cr(VI) effects already exists, but the exact mechanisms underlying Cr(VI)-induced toxicity and carcinogenicity remain unclear [54,63]. This situation results from the difficulty to access lung tumor tissue from exposed workers, making almost impossible the monitoring of molecular changes during the progression of the disease. As a result, further studies with adequate model systems and exposure regimens to the carcinogenic agent are urgently needed [63].

Concerning the model system, the most promising choices are *in vivo* models, such as mice and rats. In this case, the restricted access to the bronchial bifurcations premains a problem, leaving as the only alternative the animal sacrifice for collection of the modified cells. This raises several problems both ethical and economical. To overcome these problems, cellular systems that mimic the biological process should be used. Taking into account that Cr(VI) exerts its effects mainly in the lung [60,61], primary epithelial lung cells are the most promising system. However, to evaluate the chronic effect of Cr(VI), cells must be subjected to chronic administrations, which requires a cell system with a relatively prolonged lifespan. Thus, the primary epithelial cultures should be optimized, by a transformation process, in order to prolong their lifespan [63]. For instance, the bronchial epithelial airway system 2B (BEAS-2B) cell line is an immortalized cell line derived from normal human bronchial epithelial (NHBE) cells through infection with an adenovirus 12-simian virus 40 (SV40) hybrid virus [100]. The transformation process is possible because these cells produce a SV40 T antigen that binds to

the tumor suppressor proteins p53 and RB, prolonging the cells' life span [101, 102]. Nevertheless, this cell line maintains some characteristics of NHBE cells [103]. Few studies have used this cell system or a similar NHBE cell line [85,97,104,105], but instead, many were conducted in cells derived from other tissues or in established malignant cells [106-108]. Established malignant cells, such as HeLa cells, have particular phenotypes, namely metabolic modifications that are strongly based on glycolysis [4], and this could lead to misinterpretations of the results.

Another important aspect to take into account is the type of media used in culture procedures, especially with metabolic experiments. BEAS-2B cells respond to serum by ceasing cell division and undergoing terminal squamous differentiation [103] and many studies are performed with media supplemented with serum. Commercial medium MEM contains several components that can reduce Cr(VI) immediately after its solubilisation, thus reducing the levels of the metal that is actually in contact with the cells [109]. Borthiry and collaborators (2008) [110] studied the presence of Cr(V), a product of Cr(VI) reduction in two cultures media (DMEM and LHC-9). After dissolution of Na_2CrO_4 in the culture media, and in the absence of cells, Cr(V) was detected by ESR spectroscopy just in the DMEM medium.

The absence of serum from the culture media leads us to a delicate situation, since it is the main source of Asc in most culture media and Asc levels are rapidly diminished [111]. Considering that human cells seem to use Asc as the main reducer of Cr(VI) [72], its absence could lead to distorted results.

Another important aspect is the selected exposure regimen, since different concentrations of Cr(VI) may induce different cellular responses. The amount of Cr(VI) administered to the cell models should be sub-cytotoxic or mildly cytotoxic, in order to mimic occupational exposures to this agent. The workers that are in close contact with Cr(VI)-containing compounds are mostly exposed to low concentrations of this agent. Thus, *in vitro* studies should mimic this situation by using sub-cytotoxic concentrations and paying attention to the accumulation of Cr(VI) in the lung bifurcations of workers [63]. Actually, high levels of Cr(VI) are unlikely to induce carcinogenesis, since they may obstruct DNA replication through inhibition of polymerase activity [112,113]. On the other hand, lower levels of Cr(VI) can actually stimulate the activity of DNA polymerase. In one study where lower doses were used (up to 5 μM), an increase in the activity of this enzyme was detected, which may contribute to Cr(VI)-induced mutagenesis [112]. For instance, Alpoim and collaborators (2009) confirmed malignization by continuous exposure of BEAS-2B cells to a slightly cytotoxic concentration of Cr(VI) (1 μM), followed by low density cultivation [114].

1.4) Objectives of the Present Research Work

For many years Cr(VI) has been recognized by IARC as carcinogenic agent, and chronic exposure to Cr(VI)-containing compounds is linked to an increased risk for lung cancer development [52]. However, the exact mechanisms of Cr(VI)-induced carcinogenesis have not been fully established. In particular, the modifications of cellular metabolism, an established hallmark of cancer cells, during tumor progression and triggered by Cr(VI) are not dissected [63]. Moreover, the risk for developing lung cancer is associated with chronic exposure, but *in vitro* studies shown that Cr(VI) induce cellular modifications even after just a single insult [85, 105].

The main goal of this research project was to establish the effects of Cr(VI) acute exposures in some parameters of the energy metabolism of human bronchial epithelial cells. For that purpose, BEAS-2B cells, a non-malignant transformed NHBE cell line, was exposed to a single sub-cytotoxic concentration of Cr(VI) and the modifications of some metabolic parameters were evaluated. These parameters were the OCR, the glycolytic flux, the amount of ATP produced and levels of proteomic markers of glycolysis and mitochondria function. We aimed to determine the bioenergetic signature of BEAS-2B cells immediately after a single exposure to Cr(VI). The Cr(VI) cytotoxicity was evaluated by several methodologies, including the determination of the levels of ROS, protein carbonylation and clonogenic potential.

Chapter 2
MATERIALS AND METHODS

For specific information about the aqueous solutions used and the suppliers of chemicals and material please see the supplemental material.

2.1) Materials

2.1.1) BEAS-2B Cell Line

BEAS-2B cells (Cat. No.: 95102433) were obtained from the European Collection of Cell Cultures (ECACC). BEAS-2B cells were derived from normal bronchial epithelium obtained in an autopsy of a non-cancerous individual. In order to immortalize the primary culture, cells were infected with a replication-defective SV40/adenovirus 12 hybrid and cloned [115]. BEAS-2B cells, growing as monolayers (Fig 2.1) retain the ability to undergo squamous differentiation in response to serum. This ability can be used for screening chemical and biological agents inducing or affecting differentiation and/or carcinogenesis. The cells must be subcultured before reaching a high confluency, because highly confluent cultures rapidly undergo squamous terminal differentiation [115]. The supplier recommends BEGM culture medium, also known as LHC-9 with modification [115]. LHC-9 is composed of basal LHC medium, as described in [116,117], supplemented with several growth factors, such as epidermal growth factor, ethanolamine, phosphoethanolamine, bovine pituitary extract [118]. LHC-9 is a serum-free medium [116-118].

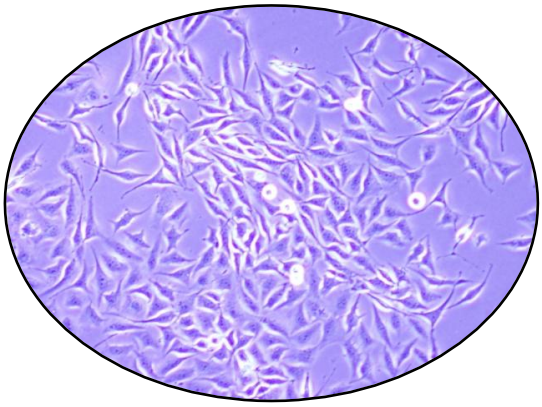

Fig. 2.1- BEAS-2B cell line used in the experimental work. Cells were in passage #13 and with four days of growing after the sub-culture procedure. Micrography acquired with a digital camera incorporated into an inverted microscope (Olympus), 100x.

2.2) Methods

2.2.1) BEAS-2B Culture Procedures

The BEAS-2B cell line was grown at 37 ^{o}C in a humidified atmosphere of 5% (v/v) CO_2. the culture recipients used were pre-coated with a coating solution [2% gelatine; phosphate-buffered saline (PBS); 2% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich)] for at least 2 h and up to 72 h. Cultures were maintained in LHC-9 growth medium (Gibco) with a depth of 200 µL/cm^2. The initial seeding density was 4,000 cells/cm^2. For each different experiment, the cell density was adapted. On routine subculture procedures, cells were harvested using a trypsin solution (Sigma-Aldrich) and collected in PBS. The resulting suspension was centrifuged at 1500 rpm for 5 min and the pellet was resuspended in a convenient volume of LHC-9 and dispersed into single cell suspensions. The number of cells was estimated using a 0.4% (v/v) trypan blue solution (Sigma-Aldrich) and a haemocytometer. To prepare cultures for experiments at least two independent counts were performed. Counts differing by more than 10% were repeated. In order to ensure that cells were in the exponential phase of growth the subculture procedures were done every 3-4 days.

2.2.2) Cr(VI) Treatments

After the seeding procedure, cells were allowed to attach to the substrate for 24 h before the addition of Cr(VI). In order to obtain final concentrations of Cr(VI) ranging between 0.1 and 2.0 µM, 10 and 100 µM Cr(VI)-containing solutions [as $K_2Cr_2O_7$ (Sigma-Aldrich)] were used. The control cultures, established and processed in parallel, received an equivalent amount of the vehicle (water).

2.2.3) Determination of Clonogenic Potential

The capability of a single cell to grow into a colony after a single Cr(VI) insult was verified according to Franken *et al* (2006) [119]. Briefly, 300 cells (between passages #14 and #18) were seeded into 6-well plates and treated with Cr(VI) (0.1 to 2.0 µM). The medium was removed and replaced with fresh and warmed Cr(VI)-free medium 24, 48 and 72 h after Cr(VI) addition. The cultures grown for nine or ten days after the seeding procedure and the colonies were fixed and stained with a colony fixation-staining solution [6.0% (v/v) glutaraldehyde; 0.5% (w/v) crystal violet (Sigma-Aldrich)] for 75 min. Next, the colonies were washed to remove the excess of dye by slowly immersing the 6-well plates into a sink filled with distilled water.

For each condition at least duplicate cultures were seeded and the number of colonies was averaged in order to determine the plating efficiency. Plating efficiency is the ratio, in percentage, between the number of colonies and the number of cells seeded. The number of colonies was counted by direct observation and all colonies stained purple were scored.

2.2.4) Determination of Protein Contents in Cellular Extracts

Cells were harvested by trypsinization as described in section 2.2.1. For protein extraction, cells were resuspended in a lysis buffer [1.0 M tris base, pH 8; 0.5 M ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich); 10% Triton X-100 (Merck); protease inhibitor (Roche)] and centrifuged at 11,000 g for 15 min at 4 ^{o}C. The supernatant containing the proteins was transferred to new centrifuge tubes. Bradford reagent (Bio-Rad) was used to determine the concentration of protein according to the manufacturer's instructions [120].

2.2.5) Determination of Oxygen Consumption Rates

OCRs were determined using the Seahorse XF24 Analyzer (Seahorse Bioscience). The Seahorse XF24 Analyzer allows the determination of OCR by a non-invasive, label-free, rapid procedure. OCR is an indicator of mitochondrial activity, since the flow of electrons during OXPHOS through the respiratory chain ends up with the consumption of the terminal electron acceptor, molecular oxygen, which is reduced to water. The Seahorse XF24 Analyzer also allows the sequential delivery of four different compounds [121]. Measurements of OCR following addition of specific compounds allow the characterization of the bioenergetic profile of cells and a more comprehensive assessment of the contribution of mitochondrial activity for cellular energetics. Measurements are performed using optical fluorescent biosensors placed into XF24 analyzer microplates that detect changes in the amount of dissolved oxygen in the medium that is in close contact with the cells cultured in the microplates. The biosensors are coupled to a fibre-optic waveguide that delivers light at an excitation wavelength equal to 532 nm to detect oxygen and transmits a fluorescent signal at 650 nm to the photodetectors [122].

To assess OCRs, 20,000 cells (passage #15) were seeded into different wells of a specific 24-well tissue culture microplate. Four wells which contained only culture medium (A1, B4, C3 and D6) were used for temperature control. Cells were seeded in a two-step way, which consist of seeding the cells in 100 μL of media and adding an additional 250 μL after 2-5 h. Cells were exposed to 1 μM Cr(VI) for 48 h. On the day of the experiment, the culture medium was replaced by 700 μL of fresh Cr(VI)-free medium.

The four pneumatic drug ports of the sensor cartridge were sequentially loaded with 50 µL of LHC-9 containing 6 µM oligomycin (OL, Sigma-Aldrich), 750 µM 2,4-dinitrophenol (DNP, Sigma-Aldrich), 1 µM rotenone (Sigma-Aldrich) and 1 µM antimycin (Sigma-Aldrich). With this sequence, the bioenergetic profile of BEAS-2B untreated and exposed to 1 µM Cr(VI) was determined.

The OCR was expressed as pmol O_2/min/µg protein.

2.2.6) Determination of Lactate Levels in Culture Media

The lactate produced by BEAS-2B cells treated and untreated with Cr(VI) was measured enzymatically.

As a preliminary experiment, the initial rate of lactate production was estimated by exposing 500,000 cells (passage #11) seeded into 100 mm dishes to 1 µM Cr(VI) for 48 h. A 100 µL aliquot of medium was collected at different times (0, 30, 60, 90, 120, 180, 240, 300 and 360 min). For each collected sample, an equivalent volume of LHC-9 was added to the test plates.

For the experiment, 100,000 cells (passage #11) were seeded in quadruplicate into 6-well plates. After an exposure to 1 µM Cr(VI) for 48 h, the growth medium was replaced by fresh one with or without 6 µM OL or 750 µM DNP. After 240 min (4 h), aliquots of 200 µL were collected and cellular extracts were prepared in order to determine their protein content.

Cellular sediments present in the collected medium (on both procedures) were precipitated with 6% perchloric acid (Merck). After 30 min on ice, the samples were centrifuged for 5 min at 11,000 g and 4 ^{o}C. The supernatant fractions, which contained the lactate, were neutralized with 20% KOH (Merck) and centrifuged at 11,000 g and 4 ^{o}C for 5 minutes to precipitate the $KClO_4$ salt.

The quantification of lactate present in the samples is based in the conversion of L-lactate to pyruvate by the LDH (Roche) in the presence of NAD^+ (Sigma-Aldrich). The NADH generated is spectrophotometrically detected at 340 nm (equation 2.1).

$$\text{L-Lactate} + NAD^+ \longrightarrow \text{Pyruvate} + NADH \qquad (2.1)$$

2.2.7) Determination of ATP Levels in Cellular Extracts

The levels of ATP produced by BEAS-2B cells were determined using a commercial kit (Roche) that exploits the capacity of luciferase to catalyze a light emitting-reaction. The commercial reagent contains luciferase and D-luciferin. The enzyme catalyzes the following reaction [123]:

$$ATP + D\text{-}Luciferin + O_2 \longrightarrow Oxyluciferin + PP_i + AMP + CO_2 + Light \quad (2.2)$$

The maximum emission peak of the resulting green light is set at 562 nm and can be detected with a luminometer. The light produced is directly proportional to the quantity of ATP present in the sample when the amount of luciferase and D-luciferin are not limitant [123].

To prepare the experiments, 100,000 or 150,000 cells (between passages #14 and #19) were seeded in triplicate into 6-well plates. Cells were exposed to 1 μM Cr(VI) for 48 h. Afterwards, the growth medium was replaced by fresh one with or without 6 μM OL, 750 μM DNP or 100 μM 2-deoxiglucose (2-DG, Sigma-Aldrich). To disrupt the cells and release the ATP to the aqueous fraction, 400 μL of the ATP lysis buffer [100 mM tris base, pH 7.75; 4 mM EDTA] at 95 ^{o}C were added to the cells. The cellular suspension was placed at 95 ^{o}C for 2 min and centrifuged at 11,000 g for 15 min.

ATP solutions with concentrations ranging from 1.6 to $1.6x10^{6}$ nM were used as standards. The luminescence was measured in a microplate reader (BMG Labtech) that detected the emitted luminescence.

The amount of ATP produced was expressed as mmol ATP/cell.

2.2.8) Sodium Dodecyl Sulfate (SDS) - Polyacrylamide Gel Electrophoresis (PAGE) and Western Blot

2.2.8.1) SDS-PAGE

Proteins obtained from cellular extracts prepared after an exposure to Cr(VI) for 48 h were fractionated into SDS-PAGE following Laemmli's method [124]. The protein samples were loaded into 9% polyacrylamide gels and separated using the electrophoresis buffer [150.0 mM tris base; 3.4 mM SDS; 192.0 mM glycine (Merck)]. Protein calibration was done using a protein mixture of known molecular weight (Amersham).

2.2.8.2) Western Blot

The fractionated proteins were transferred to polyvinylidene fluoride (PVDF) membranes. The electrotransference was performed for 1 h at 100 V and 4 °C, using an electrotransference buffer [48 mM tris base; 20% (v/v) methanol (Sigma-Aldrich); 39 mM glycine (Merck)]. PVDF membranes were blocked during 1 h with a membrane blocking solution [5.0% (w/v) nonfat dry milk (Nestlé); 150.0 mM NaCl; 50.0 mM tris base (Sigma-Aldrich); 0.1% (v/v) tween 20 (Merck)]. The primary and secondary antibodies used are detailed on Table 2.1.

Table 2.1- Primary and secondary antibodies used on western blot procedures.

Antibody		Type	Supplier	Dilution factor	Preparation
Primary (1h incubation)	Hsp60 (mouse)	Monoclonal	Enzo Life Sciences	1:20,000	Diluted in 3% (v/v) BSA in a 2 mM sodium azide solution (Sigma-Aldrich)
	GAPDH (mouse)	Monoclonal	Abcam	1:20,000	
	βF1 (rabbit)	Polyclonal	Obtained as described in [47]	1:20,000	
Secondary (45 min incubation)	Goat anti-rabbit	Polyclonal	Nordic Immunology	1:5,000	Diluted in the membrane block solution
	Rabbit anti-mouse	Monoclonal	Nordic Immunology	1:5,000	

Immunoblots were developed upon incubation with the enhanced chemiluminescence (ECL) reagent (Gibco) based on the oxidation of luminol by the peroxidase-secondary antibody conjugated enzyme. Luminol is converted to a light-emitting form at 428 nm.

The quantification of the intensity of the bands was presented as the mean of relative expression normalized to an intern control of gel (HCT116 cells) and to the mean value of respective control.

2.2.9) Determination of Oxidative Stress

The radical species produced by BEAS-2B cells were detected by flow cytometry. 100,000 cells (passage #16) were seeded in quadruplicate into 6-well plates. Cells were exposed to 1 μM Cr(VI) for 48 h, treated with 6 μM OL and harvested as described on section 2.1.1.

To detect the amount of radical species produced by cultures of BEAS-2B, the commercial dye 2'-7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Molecular Probes) was used. DCFH-DA can cross cellular membranes and, once inside cells is cleaved by esterases. A

relatively polar and impermeable product, 2',7'-dichlorodihydrofluorescein ($DCFH_2$), is formed. Through an oxidative reaction, $DCFH_2$ is converted to the fluorescent product, 2',7'-dichlorofluorescein (DCF). Accumulation of DCF monitors the redox state of cells and the fluorescence can be detected at 530 nm when the sample is excited at 485 nm. The fluorescence detected is assumed to be proportional to the concentration of H_2O_2 in cells [125].

After an exposure to OL for 1 h, cellular pellets were ressuspended in 300 μL of 5 μM DCFH-DA solution and were placed at 37 ^{o}C for 30 min (protected from light). The cellular suspensions were centrifuged at 1500 rpm for 5 min and the resulting pellets were dispersed into 200 μL of fluorescence-activated cell sorting (FACS) solution [1.0% foetal bovine serum (FBS, Gibco); 0.1% sodium azide in PBS]. Propidium iodide (PI, Sigma-Aldrich), 1 μL, was added to each sample to detect dead cells.

The fluorescence emitted by DCF and PI was detected through the FL1 and FL3 channels (BD FACSalibur), respectively, and at least 10,000 events were recorded.

2.2.10) Determination of Protein Carbonylation Levels

To detect protein carbonyls on BEAS-2B cells, the commercial kit OxyBlot (Millipore) was used. The kit contains 2,4-dinitrophenylhydrazine (DNPH) that derivatizes the carbonyl groups to 2,4-dinitrophenylhydrazone (DNPhydrazone). The derivatized proteins can be separated by gel electrophoresis and visualized through immunoblotting. As primary antibody, the kit contains a rabbit anti-DNP antibody. The secondary antibody is a horseradish peroxidase-antibody (goat anti-rabbit antibody) and the detection is done with the ECL reagent. The kit also provides a neutralization solution and a mixture of standard proteins with attached DNPhydrazone residues [126].

To determine the levels of protein carbonylation, 250,000 cells (passage #16) were seeded into 60 mm dishes and treated with Cr(VI) for 48 h as described in section 2.2.2. Cells were harvested and lysed in the lysis buffer containing 5 mM dithiothreitol (reducing agent, Sigma-Aldrich) to avoid undesired oxidation of proteins during lysis.

The derivatization mixture contained the protein sample (volume equivalent to 15 μg of protein), 10% SDS solution (to denature the proteins) and the DNPH-containing solution. The reaction was performed for 15 min at room temperature. Afterwards, the neutralization solution was added to the prepared mixtures. In parallel, two samples containing the molecular weight marker and the mixture of standard proteins were prepared. The mixtures

were loaded into a 9% running polyacrylamide gel and the electrophoresis was performed as described on section 2.2.7. The incubation with the primary antibody was performed overnight and its dilution was 1:150. The dilution used with the secondary antibody was 1:300.

As a loading control, the PVDF membrane was incubated with a monoclonal mouse primary antibody for tubulin (1:5,000; Sigma-Aldrich) for 1 h. The detection of this protein was performed as described before (See Table 2.1).

2.2.11) Statistical Analysis

The results obtained (except for the clonogenic assay) were statistically analysed with the unpaired Student's t test, using the Excel software. The results are represented as the mean ± standard error of the mean (SEM). Differences with a $P < 0.05$ and $P < 0.001$ were considered statistically significant.

Multiple variances (time and concentration) present in the clonogenic assays were statistically analysed with the two-way ANOVA model and the Bonferroni's post hoc test. The results are represented as the mean ± SEM. $^*P < 0.05$ was considered statistically significant. The analysis was performed with the Graphpad Prism Software.

Chapter 3
RESULTS

3.1) Short-Term Exposure to Sub-Cytotoxic Concentrations of Cr(VI) has no Effect in the Clonogenic Potential of BEAS-2B Cells

In a previous study performed by our group [85], the cytotoxic effects of Cr(VI) at low concentrations were assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay and testing the effects of different concentrations of Cr(VI) (0.1 to 4.0 µM) for 24, 48 and 72 h exposures. The results obtained showed a dose-dependent cytotoxic effect for Cr(VI), with a clear threshold of toxicity between 2 and 4 µM. For the low cytotoxicity concentrations (≤ 2 µM), increasing the incubation time from 24 to 48 and then to 72 h did not produce a further decrease in the viability of the treated cultures, but rather the reverse. The fact that the relative number of viable cells actually increased when the incubation time was extended indicated that those cells that survived increased their proliferating rates, suggesting a mitogenic effect for Cr(VI) at low levels. To further investigate the effects of Cr(VI), the colony-forming capacity of BEAS-2B cells exposed to low Cr(VI) concentrations exposure was analysed (Fig 3.1).

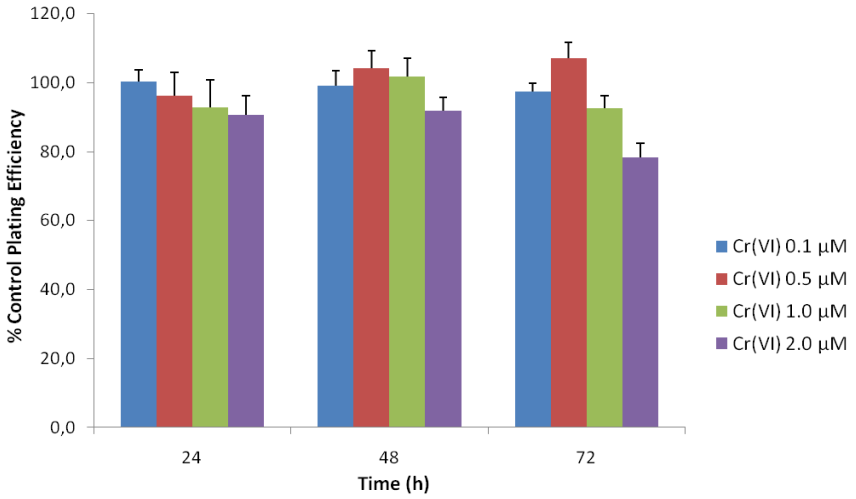

Fig. 3.1- Short-term exposure to Cr(VI) at sub-cytotoxic concentrations has no statistically significant effect in the colony forming potential of BEAS-2B cells. 300 cells were seeded and exposed to 0.1, 0.5, 1.0 and 2.0 µM Cr(VI) concentrations for 24, 48 and 72 h. The clonogenic potential of BEAS-2B cells was determined by counting the number of colonies formed after fixation and staining. Multiple comparisons by analysis of variance were done with two-way ANOVA model and the post hoc Bonferroni's test confirmed that there were no significant differences ($P > 0.05$). The results shown are the mean ± SEM of 3 independent experiments.

The clonogenic potential of BEAS-2B cells was not statistically significant affected by an acute exposure to Cr(VI) at low concentrations. In fact, the capacity of Cr(VI) treated-cells to form colonies was much similar to that of control cells. Though, exposure to 0.5 µM Cr(VI)

seemed to induce a slight increase in the clonogenic potential of cells over time (24 for 48 h and then to 72 h). On the other hand, the highest Cr(VI) concentration (2 μM) for the longest exposure, appeared to be a slightly cytotoxic and the platting efficiency diminished when compared to control (Fig. 3.1). In this case, the clonogenic potential of BEAS-2B cells seems to be slightly affected (P = 0.0523) by time exposure (Fig. 3.1).

A comparison of the results obtained in the MTT [85] and in the clonogenic assay, suggests that lower Cr(VI) levels specifically killed those cells in culture that exhibited the lowest clonogenic capacities and/or increased the clonogenic survival of those cells that resisted the acute insult.

Overall, these results led us to chose the 1 μM concentration at 48 h to further investigate the effects of Cr(VI) on the energy metabolism of BEAS-2B cells.

3.2) Short-Term Exposure to 1 μM Cr(VI) Alters the Oxygen Consumption Rate and the Maximum Respiratory Rate of BEAS-2B Cells

In order to better understand the effects that short exposures to 1 μM Cr(VI) had on the energy metabolism of BEAS-2B cells, the rates of oxygen consumption were determined. OCRs were assessed using the XF24 Extracellular Flux Analyzer, an instrument that allows the determination of OCR in adherent live cells [127].

To determine the bioenergetic profile of BEAS-2B cells, the basal OCR was measured, followed by the sequential addition of OL, DNP, rotenone and antimycin (Fig. 3.2). This specific sequence allows the determination of OL-sensitive respiration, the maximal respiration and oxygen consumption due to the activity of complexes I and complex III, respectively [128].

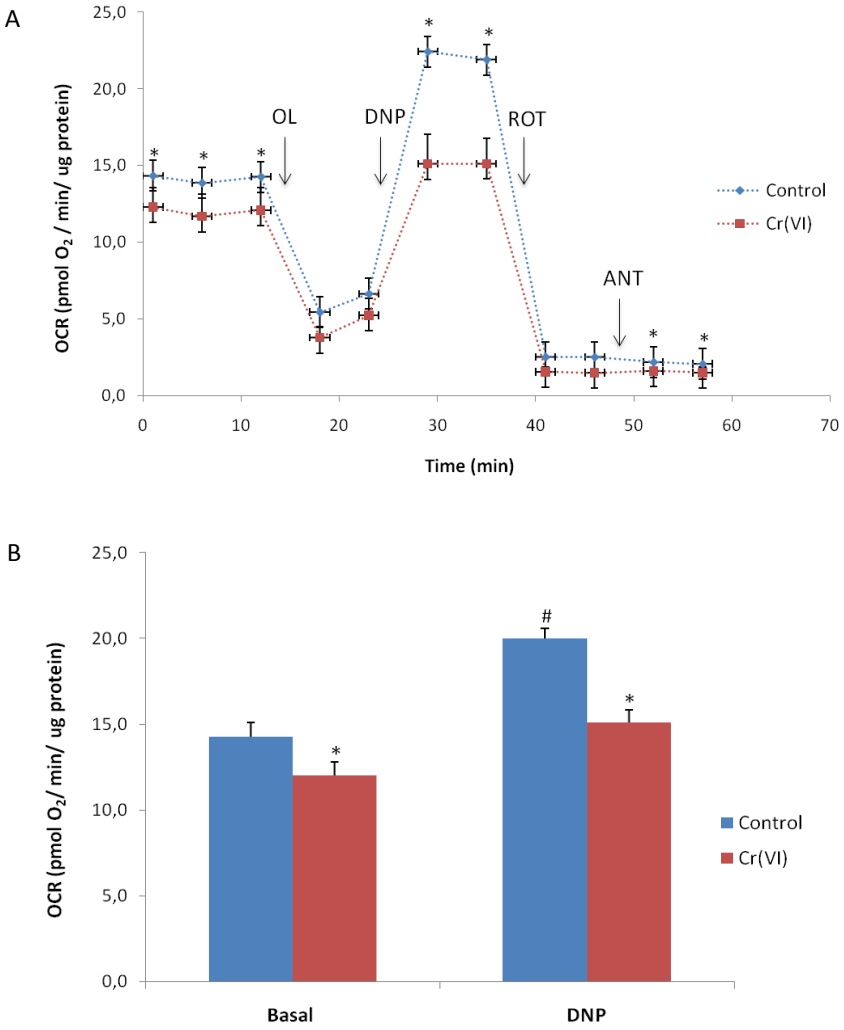

Fig. 3.2- Short-term exposure to 1 µM Cr(VI) induces a decrease in the OCR of BEAS-2B cells. 20,000 cells were seeded and treated with Cr(VI) for 48 h. (A) Bioenergetic profile obtained after real-time analysis of OCR in XF24 Extracellular Flux Analyzer of untreated and 1 µM Cr(VI)-treated BEAS-2B cells. The arrows represent the sequential addition of 6 µM OL, 750 µM DNP, 1 µM ROT and 1 µM ANT. (B) Histograms representing the OCR in basal conditions and after DNP addition. Data showed are the mean ± SEM for 8 determinations. *P < 0.05 when compared with control values by Student's t test; $^{\#}$P < 0.05 when compared with the respective basal value by Student's t test. Abbreviations: OCR, oxygen consumption rate; OL, oligomycin; DNP, 2,4-dinitrophenol; ROT, rotenone; ANT, antimycin.

Cr(VI)-treated cells and control cells have a similar bioenergetic profile (Fig. 3.2A), but the former revealed a significantly lower basal and uncoupler-stimulated OCRs. The OCR diminished after OL injection, but no significant differences were obtained between treated and untreated cultures. Very interestingly, cells exposed to Cr(VI) did not show statistically significant differences between the basal and the maximal respiratory rates (after DNP injection), whereas control cells did. The mean value of OCR in control cells was approximately 20 pmol O_2/min/µg protein, whereas Cr(VI)-treated cells displayed an OCR of about 15 pmol O_2/min/µg protein (Fig 3.2B – DNP bars). Overall, the results indicate that Cr(VI) is interfering with the maximum respiratory capability of cells. Rotenone induce a large decrease in the OCR,

both in control and Cr(VI)-exposed cultures, setting the values of OCR close to zero (2.21±0.23 and 1.89±0.13 pmol O_2/min/µg protein for control and Cr(VI)-exposed cells, respectively).

3.3) Short-Term Exposure to 1 µM Cr(VI) Alters the Basal and the Maximal Glycolytic Flux of BEAS-2B Cells

To further investigate the effect of short-term exposures to 1 µM Cr(VI) in the glycolytic flux of the cells, the levels lactate in the culture media were measured after 48 h of Cr exposure. In order to assess the initial rate of lactate production, a preliminary experiment was done, in which the release of lactate into the medium was followed for 360 minutes (Fig. 3.3A). Afterwards, the levels of lactate production were determined at a selected time point (240 min) after treatment with OL and DNP (Fig. 3.3B).

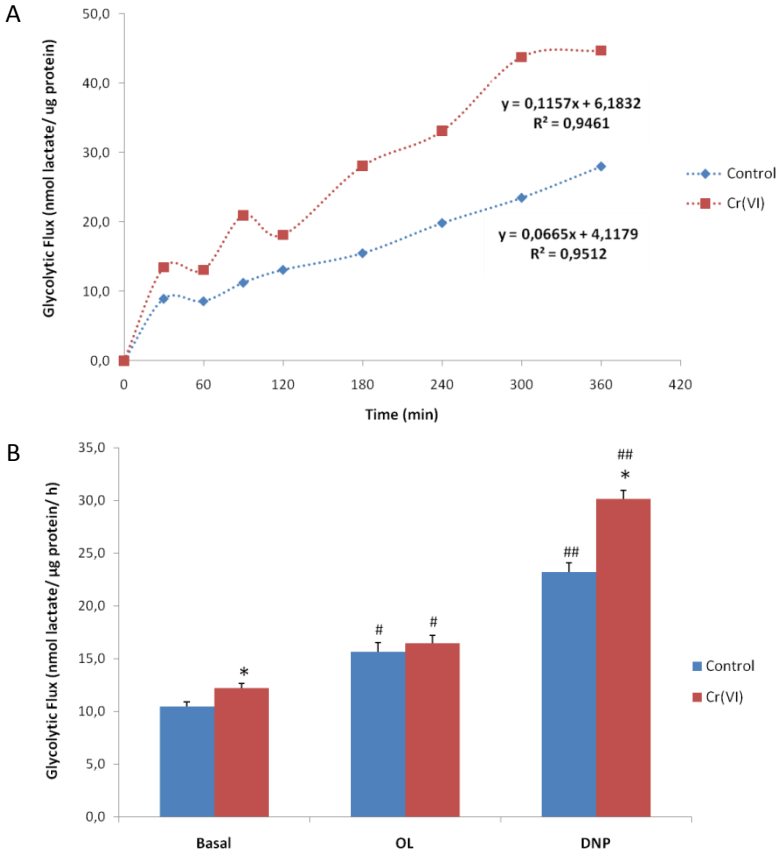

Fig. 3.3- Short-term exposure to 1 µM Cr(VI) stimulates the initial rate of lactate production and increase basal and maximum glycolytic fluxes. (A) 500,000 cells were exposed to 1 µM for 48 h. Lactate levels were assessed enzymatically as described under Material and Methods. (B) 100,000 cells were exposed to 1 µM for 48 h and the concentration of lactate in the medium was determined enzymatically 4 h after treatment of cells with 6 µM OL (4 h) and 750 µM DNP (1 h). (B) The results shown are the mean ± SEM of 4 determinations. *P < 0.05 when compared with control values by Student's t test; $^{\#}$P < 0.05 and $^{\#\#}$P < 0.001 when compared with the respective basal value by Student's t test. Abbreviations: OL, oligomycin; DNP, 2,4-dinitrophenol.

Both control and Cr(VI)-exposed cells showed roughly a linear production of lactate during the first 6 h (Fig. 3.3A). Short-term exposure of BEAS-2B to this sub-cytotoxic concentration of Cr(VI) significantly increased the basal rate of lactate production when compared with controls (Fig. 3.3B). Inhibition of the H^+-ATP synthase with OL significantly increased the rates of aerobic glycolysis in both control and Cr(VI)-treated cells (Fig. 3.3B). Cr(VI) treatment increased significantly the maximum lactate production rates as determined in the presence of the mitochondrial uncoupler DNP. Moreover, a higher maximum glycolytic flux was observed in Cr(VI)-treated cells when compared with controls (Fig. 3.3B). Overall, these results demonstrate that short-term treatment of Cr increases the glycolytic flux of human bronchial epithelial cells.

3.4) Short-Term Exposure to 1 µM Cr(VI) had no Effects in the Levels of ATP of BEAS-2B Cells

The levels of ATP produced by BEAS-2B cells were determined after a 48 h exposure to Cr(VI) and in control cells (Fig. 3.4).

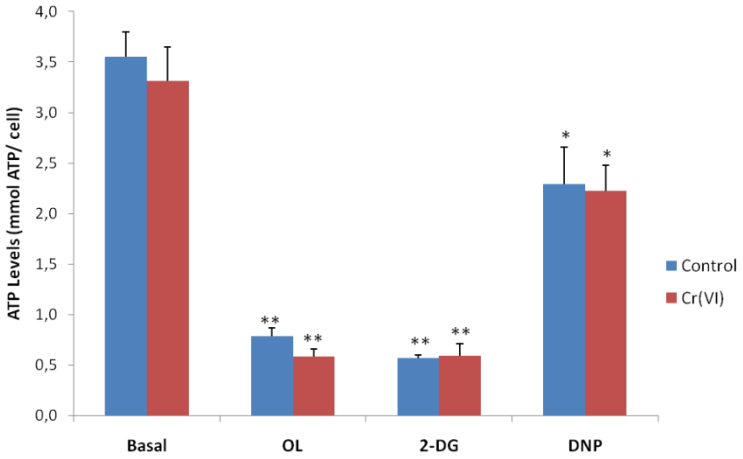

Fig. 3.4- Short-term exposure to 1 µM Cr(VI) does not significantly affect the production of ATP. BEAS-2B cells were exposed to 1 µM Cr(VI) for 48 h. Cells were then treated with the following agents: 6 µM OL, 750 µM DNP and 100 µM 2-DG, or left untreated (basal) and the intracellular concentration of ATP was determined. The histograms represent the means ± SEM of 3 independent experiments. *P < 0.05, and **P < 0.001, when compared with the respective basal value by Student's t test, respectively. Abbreviations: ATP, adenosine triphosphate; OL, oligomycin; DNP, 2,4-dinitrophenol; 2-DG, 2-deoxyglucose.

The exposure of BEAS-2B cells to Cr(VI) for a 48 h period did not affect the intracellular basal ATP levels (Fig. 3.4). However, a significant and similar drop in cellular ATP levels was

observed when aerobic glycolysis was inhibited with 2-DG or when the H^+-ATP synthase was inhibited with OL (Fig. 3.4). Overall, these data suggest that glycolysis and OXPHOS contributed equally to energy production in BEAS-2B cells. Interestingly, we observed that DNP promoted a slight drop in ATP levels in both control and Cr(VI)-treated cells, when compared with OL or 2-DG-treated cells. These findings indicate that the large stimulation of glycolysis triggered by DNP treatment (see Fig. 3.3B) compensates the energetic imbalance imposed by uncoupling energy transduction in the mitochondria.

3.5) Short-Tem Exposure to 1 µM Cr(VI) Alters the Expression of Glycolytic and Mitochondrial Proteins and the Bioenergetic Signature of BEAS-2B Cells

The levels of the cytosolic GAPDH protein and the mitochondrial Hsp60 and βF1 proteins were determined in control and in treated cells with Cr(VI) to for 48 h (Fig. 3.5).

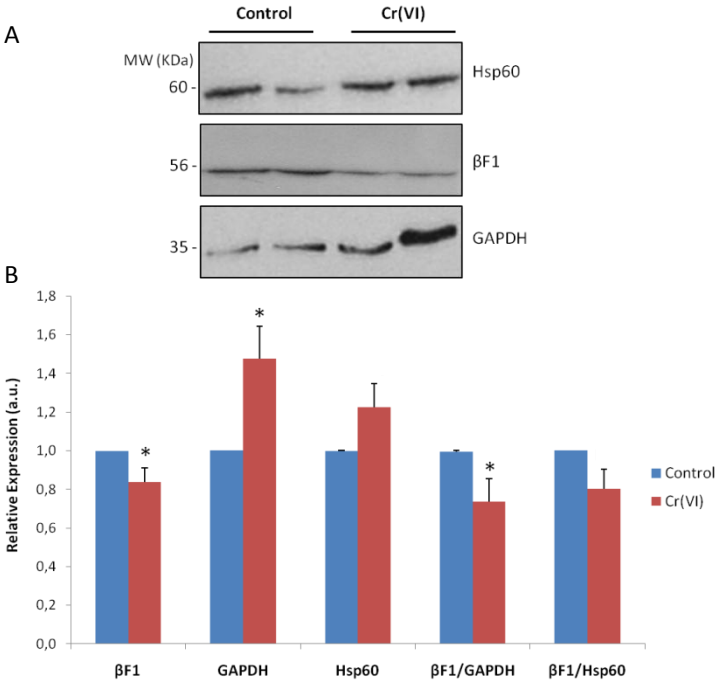

Fig. 3.5- Short-term exposure to 1 µM Cr(VI) alters the expression of glycolytic and mitochondrial markers. (A) Representative western blot of the expression of Hsp60, βF1 and GAPDH in two different preparations of untreated and 1 µM Cr(VI)-treated cells with for 48 h. (B) Histograms representing the relative expression (mean ± SEM) of βF1 (n=14), GAPDH (n=13) and Hsp60 (n=11) and the βF1:GAPDH (n=14) and the βF1:Hsp60 (n=11) ratios. The expression was normalized to an internal control (extracts of HCT116 cells) assayed in all the gels and expressed relative to the mean value of untreated cells. *P < 0.05 when compared with control conditions by Student's t test. Abbreviations: Hsp60, heat shock protein 60; βF1, β-F1-ATPase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MW, molecular weight.

Consistent with glycolytic flux and OCR data, the expression of GAPDH was significantly increased in Cr(VI)-treated cells (Fig. 3.5). Moreover, treatment of cells with Cr(VI) resulted in the down-regulation of the βF1 catalytic subunit of the H^+-ATP synthase. Changes in the expression of βF1 occurred in the absence of statistically relevant changes in the expression of the structural mitochondrial protein Hsp60. Therefore, the ratio βF1/GAPDH (bioenergetic signature) was significantly diminished in Cr(VI)-treated cells. No significant changes were observed in the βF1/Hsp60 ratio (Fig. 3.5B).

3.6) Short-Term Exposure to 1 µM Cr(VI) Induces the Production of Oxidative Stress after Oligomycin Treatment

In order to further investigate the toxicity that Cr(VI) may induce in BEAS-2B cells, we analysed the production of oxidative stress by flow cytometry (Fig. 3.6).

Treatment of BEAS-2B cells with 1 µM Cr(VI) did not interfere with the basal production of radical species (Fig. 3.6B). However, whereas the treatment with OL did not promote significant changes in the production of radical species in control cells, Cr(VI)-treated cells showed a significant increase in the levels of oxidative stress after the addition of OL (Fig. 3.6B).

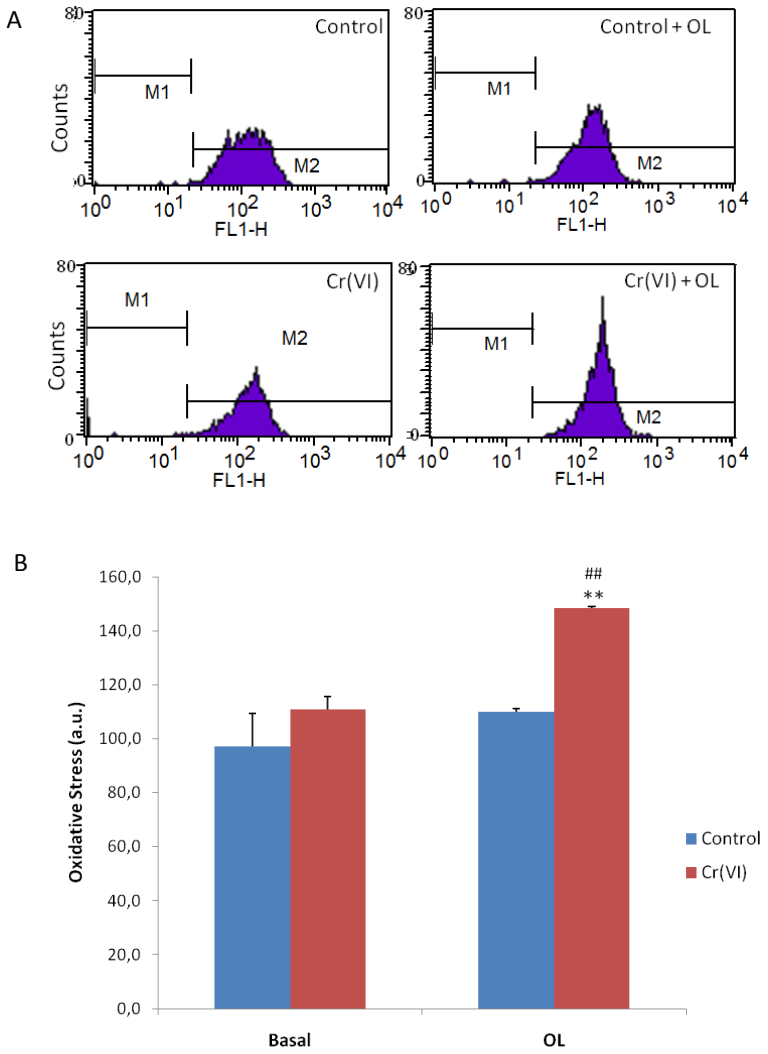

Fig. 3.6– Short-term exposure to Cr(VI) induces the production of oxidative stress in OL-treated cells. (A) 100,000 cells were seeded and exposed to 1 µM Cr(VI) for 48 h. Cells were treated with OL for 1 h and the oxidative stress was detected by flow cytometry. At least, 10,000 events were recorded. (B) The histograms represent the means ± SEM. **P < 0.001 when compared with control values by Student's t test; $^{\#\#}$P < 0.001 when compared with the respective basal value by Student's t test Abbreviations: OL, oligomycin.

Free radicals that are not detoxified by the cellular defences can attack and modify components, such as nucleic acids, lipids and proteins [129]. Consistent with the lack of differences in basal ROS production between control and Cr(VI)-treated cells, we observed that cellular proteins carbonylation was altered by treatment with 1 µM Cr(VI) (Fig. 3.7A). The quantification of p50, a representative band to a 50 KDa protein (Fig. 3.7B), supported the absence of significant differences between Cr(VI)-treated and control cells.

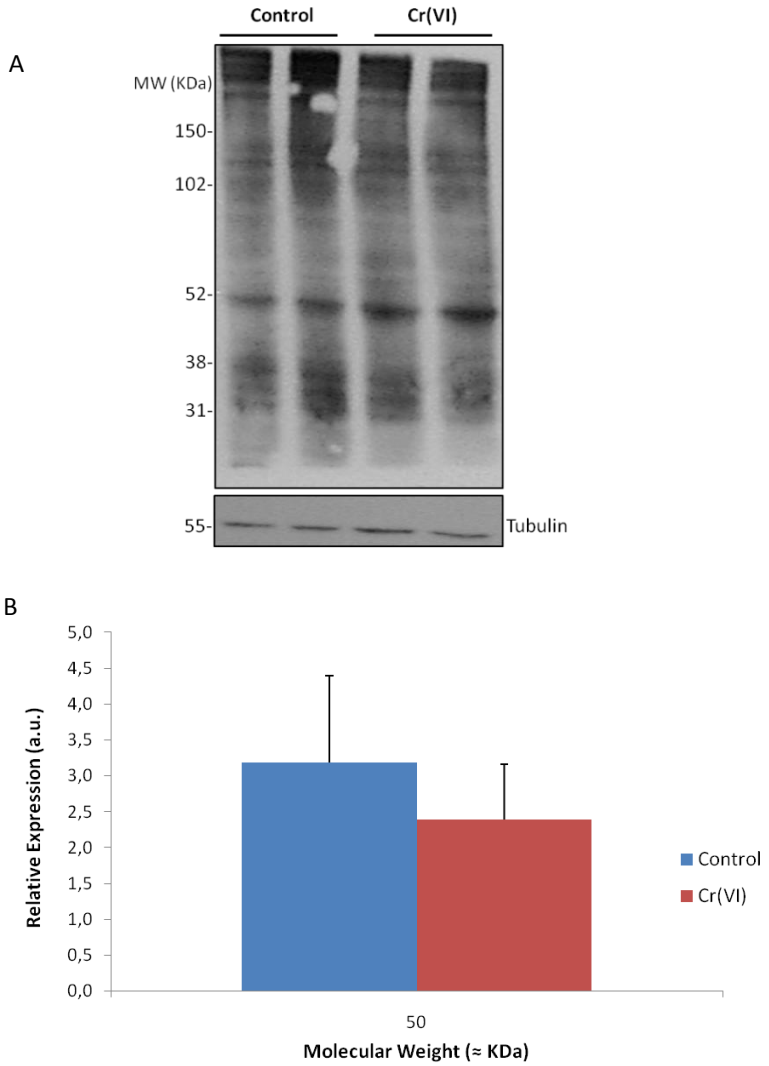

Fig. 3.7- Short-term exposure to 1 µM Cr(VI) does not alter the levels of carbonylation of cellular proteins. (A) Representative western blot of the levels of protein carbonyls in two different preparations of untreated and treated cells with 1 µM Cr(VI) for 48 h. (B) Histogram representing the mean ± SEM of the relative expression (normalized with tubulin expression levels) of the carbonyls detected in the protein with a molecular weight of 50 KDa (n=4). *P < 0.05 when compared with control conditions by Student's t test. Abbreviations: MW, molecular weight.

Chapter 4
DISCUSSION

For a long time, Cr(VI) has been established as a carcinogenic agent, predisposing primarily to squamous lung cancer [52]. Nevertheless, the mechanisms underlying Cr(VI)-induced carcinogenesis are not fully understood. Most studies focused mainly on the genotoxic characteristics of Cr(VI) and few have explored the possible modifications induced by Cr(VI) on the cellular energy metabolism after short-term exposure to the metal. This poses a problem, since the deregulation of cellular energetics in lung cancer is a hallmark fulfilled by more than 97% of both squamous and adenocarcinomas of the lung [48,51].

To better understand the effects of Cr(VI) on lung cells we chose a transformed NHBE cell line to conduct the metabolic studies. BEAS-2B is an immortalized and non-tumorigenic cell line derived from normal human epithelium that strongly resembles the main *in vivo* target of Cr(VI) carcinogenicity [103,115]. The concentrations of Cr(VI) used in our experiments were chosen in an attempt to mimic the levels of soluble Cr(VI) concentrations found in the lungs of chromate workers exposed. Tsuneta and colleagues (1980) found in peripheral lung tissues a mean Cr(VI) concentration of about 40 μg per g wet weight in chromate workers with lung cancer [60]. According to Caglieri *et al* (2008), the concentrations applied by us in BEAS-2B cells are comparable to the levels found in those lungs [105]. Therefore, we suggest that the results obtained in our *in vitro* studies can be useful to better understand the effects of Cr(VI) in exposed workers.

To explore the cytotoxicity of Cr(VI) at low levels in BEAS-2B cells we performed a clonogenic assay, testing different concentrations and several exposure periods (Fig. 3.1). None of the concentrations significantly affect the potential of cells to form colonies. The platting efficiency of cells exposed to 2 μM Cr(VI) for 72 h slightly decreased, suggesting that this concentration may be cytotoxic. In a previous study [85] where the viability of cells was tested with the MTT assay, the threshold of Cr(VI) toxicity was also set between 2 and 4 μM. Interestingly, lower levels of Cr(VI) appeared to increased the viability of cells [85]. In the clonogenic assay, lower levels did not interfere with the capability of cells to form colonies, suggesting that Cr(VI) may increase the clonogenic survival of cells more resistant to it, or that it selectively may kills the cells with lower potential to form colonies. In other studies, 16 h Cr(VI) treatments with 2.5 and 5 μM doses resulted in a marked decrease in the clonogenic survival of cells [99]. Higher levels of Cr(VI) are unlikely to induce carcinogenesis, due to polymerase arrest, whereas lower levels may stimulate the activity of the enzyme [112,113]. Overall, the mutagenic and transforming actions of Cr(VI) may occur at very low levels (< 2 μM) of exposure.

The subsequent studies aimed to investigate the effects of the metal on energy metabolism of BEAS-2B cells were performed with a single concentration of Cr(VI), 1 μM. The levels of Cr(VI) decreased in the culture medium, over time, due to its fast transport through the anion carrier [67]. Considering this situation, the different analyses carried out were not performed after long time-period of incubation with Cr(VI) (72 h), because many cells may not even be in contact with Cr(VI), and the effects of the metal will be diluted. Moreover, shorter times of incubation also prevented an excessive degree of confluence of cultures and the entrance into the stationary phase due to contact inhibition. An exposure period of 24 h was considered insufficient to observe modifications in some of the analysed parameters, particularly, in the expression of proteins. So, we decided to treat cells with 1 μM Cr(VI) for 48 h.

The results clearly showed that a single insult of 1 μM Cr(VI) interferes with the activity of mitochondria in these human bronchial epithelial cells. The bioenergetic analysis with the XF24 Extracellular Flux Analyzer demonstrated that Cr(VI) diminished the consumption of O_2 in BEAS-2B cells by interfering with both the basal and uncoupler stimulated respiratory activity of mitochondria (Fig. 3.2). These changes occurred concurrently with an increase in the glycolytic flux. The effect of Cr(VI) toxicity in the energy metabolism is better illustrated by the large differences observed in aerobic glycolysis of the cells subjected to stressful conditions, such as when the cells were incubated in the presence of the uncoupler DNP (Fig. 3.3). Moreover, proteomic analysis of both glycolytic and mitochondrial proteins confirmed these findings showing a diminished expression of βF1 concomitant with an increased GAPDH (Fig. 3.5), which is translated into a lower overall mitochondrial potential (lower BEC Index). The analysis of the expression levels of GAPDH, Hsp60 and βF1 proteins in different types of solid tumors demonstrated that malignant cells display a lower BEC Index (βF1/GAPDH ratio) [12, 48, 49].Remarkably, our findings illustrate that a short-term exposure of bronchial epithelial cells to a single insult of 1 μM Cr(VI) triggers modifications in the energy metabolism similar to the ones found in carcinomas. The over-expression of GAPDH in Cr(VI)-treated cells correlates positively with higher rates of an aerobic glycolytic flux. Exposed cells produce more lactate than controls and have an enhanced capacity to stimulate the glycolytic flux when the activity of mitochondria is compromised.

In carcinomas a lower βF1/Hsp60 ratio was also found that indicates a reduction of the bioenergetic competence of mitochondria [12,48,49]. A single and sub-cytotoxic concentration of Cr(VI) appears not to affect the expression level of Hsp60 protein and the effects in the βF1 appears to be insufficient to interfere with the bioenergetic capacity of the cells. Consistent

with this observation is the finding that Cr(VI) did not interfere with the production of ATP in Cr(VI) treated cells, when compared with the respective basal value. In previous studies, it was demonstrated that Cr(VI) affects the capability of cultured hamster fibroblasts to produce ATP but, the doses administrated in those studies were in the millimolar range [92,130] and much higher than the one used in this study. We suggest that probably it is necessary higher doses or prolonged exposures to Cr(VI) to interfere with the capability of cells to produce their energy.

Cr(VI) treatment displays a bioenergetic signature and a shift to glycolysis, by modifying the expression of key enzymes of the energy metabolism. The effects of Cr(VI) in OXPHOS are not translated into lower levels of ATP production, but the OCR is affected, indicating that somehow Cr(VI) is interfering with the activity of the electron transport chain. Myers and colleagues (2010) have demonstrated that Cr(VI) diminished the activity of complexes I and II and of that the mitochondrial aconitase. Aconitase is a TCA cycle enzyme and its inhibition will probably slow the production of NADH, the source of electrons to complex I, which could contribute to diminish the activity of the respiratory chain [97]. Moreover, specific inhibitors of aconitase caused a decrease in the consumption of O_2 [131], reinforcing the hypothesis of the effect of Cr(VI) in interfering with the electron transport. In addition, in rat liver mitochondria Cr(VI) causes a partial inhibition of the activity of complexes I and II [94].

Many studies have related the toxicity of Cr(VI) with the production of oxidative stress inside cells [76-78]. A short exposure to 1 µM Cr(VI) did not induced the basal production of oxidative stress nor the carbonylation of proteins. Only when Cr(VI)-exposed cells were stressed by treatment with OL, they did show higher levels of oxidative stress. OL is a specific inhibitor of the ATP synthase, by blocking its proton channel. By doing so, the protons tend to accumulate in the mitochondrial inter-membrane space triggering a $\Delta\psi_m$ increase [132]. Higher values of $\Delta\psi_m$ are associated with ROS formation [7]. Probably, the intracellular levels of anti-oxidants in Cr(VI)-treated cells were altered when compared with control cells and the treatment with OL had a more pronounced effect in Cr(VI) treated cells. Some studies have demonstrated that Cr(VI) interfered with the Trx system, which is responsible for maintenance of intracellular thiol redox balance. For instance, exposure for 16 h to 5 µM Cr(VI) induced a complete oxidation of the mitochondrial Trx2 and peroxiredoxin 3 proteins [99]. It is likely that the capability of Cr(VI)-treated cells to maintain the thiol balance and detoxify free radicals is compromised in these extreme incubation conditions.

Overall, we demonstrated for the first time that a short-term exposure of human bronchial epithelial cells to a sub-cytotoxic concentration of Cr(VI) triggers a rapid remodelling of the cellular energy metabolism by affecting the mitochondrial respiratory capacity and the

enhancement of aerobic glycolysis. These findings might be at the heart of lung cancer induction/progression.

Chapter 5
CONCLUSIONS

Cr(VI) is a well-recognized carcinogenic agent and prolonged exposures are associated with development of lung cancer [52]. In this work, we were interested in determining the effects of a single exposure to Cr(VI) in the energy metabolism of human bronchial epithelial cells using as paradigm the BEAS-2B cells. We can conclude:

1) Sub-cytotoxic doses of Cr(VI) (0.5-1.0 μM) that were previously shown to have a mitogenic effect did not interfere with the colony forming potential of BEAS-2B cells.

2) 1 μM Cr(VI)-treated cells had a lower basal and uncoupler-stimulated OCR when compared with control cells.

3) 1 μM Cr(VI)-treated cells showed higher basal and stressed rates of aerobic glycolysis.

4) Cr(VI)-treated cells showed a reduction in their bioenergetic signature when compared with non-treated cells in agreement with changes in the energy metabolism.

5) 1 μM Cr(VI) treatment did not alter the basal production of oxygen radicals nor induced differences in the carbonylation of cellular proteins. Nevertheless, Cr(VI)-treated cells exposed to OL increased the production of ROS when compared with control cells.

Chapter 6
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Supplemental Material

1) Composition of Solutions Commonly used During the Experimental Procedures

All reagents and solvents used in the practical work were from analytical grade. All aqueous solutions were prepared with distilled water treated in a Milli-Q water purification system, unless specified. All aqueous solutions and material used in asepsis conditions, which were not bought sterilized, were sterilized either by moist heat sterilization for 22 minutes at 121 ^{o}C (when suitable) or by syringe with 0.2 µm filters pores.

1.1) Composition of Solutions used in Tissue Culture and Clonogenic Assay

1) 10x PBS
   - 14.7 mM KH_2PO_4
   - 81.0 mM Na_2HPO_4
   - 1370.0 mM NaCl
   - 26.8 mM KCl in water.

The pH of this solution was adjusted to pH 7.4 with 1.0 M NaOH or 1.0 M HCl solutions and stored at room temperature.

1x PBS solution was prepared from 10x PBS by adding the appropriate volume of water. This solution was subjected to moist heat sterilization and stored at 4 ^{o}C.

2) 1 M NaOH
   - Prepared by dissolving an appropriate amount of NaOH in water.

   This solution was stored at room temperature.

3) 1 M HCl
   - Prepared by diluting a 37% HCl solution with water.

   This solution was stored at room temperature.

4) 2% (w/v) Gelatin
   - Prepared by dissolving an appropriate amount of gelatin from bovine skin - type B in water.

This solution was subjected to moist heat sterilization and stored at 4 ^{o}C.

5) 2% (w/v) BSA
   - Prepared by dissolving an appropriate amount of BSA in water.

This solution was sterilized using a 0.2 µm pore size filter and stored at 4 ^{o}C.

6) Coating – solution
   - Prepared with 2% gelatin, 1x PBS and 2% BSA in the proportions of 50%, 45% and 5%, respectively.

This solution was prepared with sterile solutions and stored at 4 ^{o}C.

7) 1x Trypsin
   - Prepared by diluting a 10x trypsin stock solution from porcine pancreas (25 g/L in 0.9% sodium chloride) with 1x PBS.

This solution was prepared with sterile solutions and stored at 4 ^{o}C.

8) 1 mM Cr(VI) solution
   - Prepared by dissolving an appropriate amount of $K_2Cr_2O_7$ in water.

This solution was stored at room temperature. 100 µM and 10 µM solutions were prepared by dilution of the stock solution with water. Both solutions were sterilized using a 0.2 µm pore size filter and stored at 4 ^{o}C.

9) Colony fixation-staining solution
   - 6.0% (v/v) glutaraldehyde
   - 0.5% (w/v) crystal violet in water.

This solution was stored at 4 ^{o}C.

1.2) Composition of Solutions used in SDS-PAGE and Western Blot

1) 5x buffer
   - 1 M Tris base, pH 6.8: 25%
   - 2-Mercaptoethanol: 25%
   - 85% (v/v) Glycerol: 50%.

For 1 mL of solution, 0.1 g of sodium dodecyl sulfate (SDS) and 0.005 g of bromophenol blue sodium salt was added.

The solution was aliquoted and stored at -20 ^{o}C.

2) Running and stacking gels

9% Running gels were prepared by adding, in the order given, the following components:

- Water (1.83 mL)
- 40% (v/v) Acrylamide/bis solution (1.13 mL)
- Tris base solution (pH 8.8) (1.95 mL)
- 10% (w/v) SDS (0.05 mL)
- 10% (w/v) Ammonium persulfate (0.05 mL)
- N,N,N',N'-Tetramethylethylenediamine (TEMED) (0.003 mL).

5% Stacking gels were prepared by adding, in the order given, the following components:

- Water (2.19 mL)
- 40% (v/v) Acrylamide/bis solution (0.37 mL)
- Tris base solution (pH 6.8) (0.38 mL)
- 10% (w/v) SDS (0.03 mL)
- 10% (w/v) Ammonium persulfate (0.03 mL)
- TEMED (0.003 mL).

3) Electrophoresis buffer

- 150 mM Tris base
- 192 mM Glycine
- 3.4 mM SDS in water.

This solution was stored at room temperature.

4) Electrotransference buffer

The solution was prepared, just before use, by dilution of a 10x stock solution (1:10) in water and methanol. The final solution contained:

- 1x electrotransference buffer
    - 48 mM tris base
    - 39 mM glycine
- 20% (v/v) methanol.

5) Tris buffered saline – tween 20 (TBS-T) buffer

The solution was prepared, just before use, by dilution of a 10x TBS stock solution (pH 7.4) in water (1:10) and addition of tween 20. The final solution contained:

- 1x TBS:
  - 150 mM NaCl
  - 50 mM Tris base
- 0.1% (v/v) Tween 20.

6) Membrane block solution

- 5% (w/v) Nonfat dry milk diluted in TBS-T.

The solution was prepared just before used.

7) Primary antibodies dilute solution

- 3% BSA in a 0.002 M sodium azide solution.

This solution was prepared in aliquots and stored at -20 ^{o}C.

The diluted primary antibody solution was stored a 4 ^{o}C.

1.3) Composition of Solutions used in Lactate Levels Assessment

1) Lactate buffer

- 1.0 M Glycine
- 0.4 M Hydrazine hydrate
- 1.3 M EDTA.

The pH of this solution was adjusted to 9.5 with a NaOH solution and prepared just before use.

2) 6% (v/v) Perchloric acid

- Prepared by dilution of a 60% (v/v) perchloric acid solution.

This solution was stored at room temperature.

3) 20% (w/v) KOH

- Prepared by dissolving an appropriate amount of KOH in water.

This solution was stored at room temperature.

1.4) Composition of Solutions used to Prepare Protein Extracts

1) Protein lysis buffer
   - 1.0 M Tris base (pH 8): 0.025 mL
   - 0.5 M EDTA: 0,200 mL
   - 10% (v/v) Triton X-100: 0.250 mL.

   For a final volume of 0.475 mL half tablet of EDTA-free protease inhibitor was added.

   This solution was stored at -20 ^{o}C in aliquots.

1.5) Composition of Solutions used to Determine ATP Levels

1) ATP lysis buffer
   - 100 mM Tris base
   - 4 mM EDTA.

   The pH of the solution was adjusted to 7.75 and stored at room temperature.

1.6) Composition of Solutions used to Determine Radical Species Levels

1) 5 µM DCFH-DA
   - A 10 mM DCFH-DA solution was diluted with 1x PBS.

     The stock solution was stored at -70 ^{o}C in aliquots and the 5 µM solution was prepared just before use. The recipient was protected from light with aluminum.

1) FACS solution
   - 1.0% FBS
   - 0.1 % Sodium azide in 1x PBS.

     This solution was stored at 4 ^{o}C.

2) Suppliers

2.1) Reagents and Solutions

1) Sigma-Aldrich, St. Louis, USA, provided:

- 0.4% (m/v) Trypan blue solution (T8154) to count cells;
- DNP to treat cells;
- 2-DG (D3179) to treat cells;
- Antimycin A (A8674) to treat cells;

- Dimethyl sulfoxide (D589) to froze cells;
- Dithiothreitol (D9779) to use as a reducing agent;
- EDTA (ED2SS) to prepare multiple solutions;
- Gelatin from bovine skin - type B (G9391) and bovine serum albumin (A9418) to coat culture flasks;
- Glutaraldehyde solution (G5882) and crystal violet (C3886) to prepare colony fixation-staining solutions;
- Hydrazine hydrate (225819) to prepare lactate buffer;
- $K_2Cr_2O_7$ (P2588) to prepare Cr(VI) solutions;
- KH_2PO_4 (P5379), NaCl (S9625), Na_2HPO_4 (S0876-500G) and KCl (P5405) to prepare 10x PBS;
- Methanol (32213) to prepare western blot solutions;
- OL (O4876) to treat cells;
- PI(P4170) to detect dead cells;
- Rotenone (R8875) to treat cells;
- SDS (L5750) to prepare multiple solutions;
- Sodium azide (S2002) to prepare multiple solutions;
- Tris base (T1503) to prepare multiple solutions;
- Trypsin solution from porcine pancreas (T8154) to use in the culture cells routine;
- Tubulin antibody (monoclonal, T5168) to use on western blot procedures;
- NAD^+ (N1511) to use on lactate measurements;

2) Merck Chemicals, Darmstadt, DE, provided:

- 2-Mercaptoethanol (8.05740.0250) and 85% glycerol (1.04094.1000) to prepare 5x buffer;
- 60% Percloric acid (1.00518.1001) to prepare 6% percloric acid solutions to use on lactate measurements;
- Bromophenol blue sodium salt (111746) to prepare 5x buffer;
- Glycine (5.00190.1000) to prepare western blot solutions and lactate buffer;
- pH-indicator solution-pH 4 -10 (1.09175.0100) to monitor the pH of solutions;
- NaOH (6498.1000) to prepare the 20% NaOH solution to use on lactate measurements;
- Triton X-100 (1.12298.0101) to prepare protein lysis buffer;
- TWEEN® 20 – detergent (655205) to prepare western blot solutions;

3) Invitrogen, Barcelona, ES, provided:

- FBS (10106-169) to froze cells;
- LHC-9 medium (12680-013) to use in culture cells' routine;
- Novex® ECL chemiluminescent reagent kit (WP20005) to reveal immunoblots;
- DCFH-DA (D-399) to detect radical species formation;

4) Panreac Química, S.A., Barcelona, ES, provided:

- HCl (131020.0719) to prepare pH adjustment solutions;

5) Bio-Rad, Madrid, ES, provided:

- 40% Acrylamide/Bis Solution 29:1 to (161-0146) to use on western blot procedures;
- Ammonium persulfate (161-0700) to use on western blot procedures;
- Bio-Rad's protein assay kit (500-0006) to determine protein concentrations by Bradford method;
- TEMED (161-0801) to use on western blot procedures;

6) Roche, Madrid, ES, provided:

- ATP bioluminescence assay kit HS II (11 699 709 001) to determine ATP levels;
- EDTA-free protease inhibitor cocktail tablets (11 836 170 001) to prepare protein lysis buffer;
- LDH (10 127 230 001) to determine lactate levels on medium samples;

7) GE Healthcare, Barcelona, ES, provided:

- Amersham full-range rainbow molecular weight markers (RPN800E) to use on western blot procedures;

8) Abcam, Cambridge, UK, provided:

- GAPDH antibody (ab8245) to use on western blot procedures;

9) Enzo Life Sciences, Madrid, ES, provided:

- Hsp60 antibody (ADI-SPA-807-E) to use on western blot procedures;

10) Nordic Immunology, Madrid, ES provided:

- Peroxidase-conjugated anti-mouse (5541) or anti-rabbit secondary (6105) antibodies;

11) Seahorse Bioscience, Copenhagen, DK, provided:

- Calibration solution (100840-000) to place into the XF 24-well analyzer microplates;

12) Millipore, Madrid, ES, provided:

- OxyBlot protein oxidation detection kit (S7150) to detect the levels of carbonylation of proteins;

2.2) Material and Equipment:

1) Nalgene, New York, USA, provided:

- Mr Frosty cryo 1 ^{o}C freezing container to froze cells;

2) Corning, New York, USA, provided:

- Flasks with vented cap and test plates to tissue culture handling;

3) Orange Scientific, Braine-l'Alleud, BE, provided:

- Falcon tubes to tissue culture handling and to aqueous solutions storage;
- Flasks with vented cap and test plates to tissue culture handling;

4) BD Falcon, Madrid, ES, provided:

- Falcon tubes to tissue culture handling and to aqueous solutions storage;
- Petri dishes (multiple sizes) to tissue culture handling;
- Serologic pipettes to tissue culture handling;

5) Sarstedt, Rio de Mouro, PT, provided:

- Falcon tubes to tissue culture handling and to aqueous solutions storage;
- Serologic pipettes to tissue culture handling;

6) Prestige Medical, Blackburn, UK, provided:

- Omega autoclave to perform heat moist sterilizations;

7) Millipore, S.A., Molsheim, FR, provided:

- Simplicity^{TM} Milli-Q water purification system;

8) Bio-Rad, Madrid, ES, provided:

- Mini-PROTEAN 3® system to perform SDS-PAGE;
- Trans-Blot® Electrophoretic Transfer Cell to perform protein electrotransference;

9) KOJAIR, Vilppula, FI, provided:

- Biowizard – 100 class II safety cabinet to tissue culture handling in aseptic conditions;

10) Kodak, Madrid, ES, provided:

- Kodak-X-OMAT 2000 processor to image processing;

11) Seahorse Bioscience, Copenhagen, DK, provided:

- Seahorse XF24 Analyzer to determine OCR;
- XF 24-well tissue culture microplates to seed the cells for OCR determinations;
- XF 24-well XF24 analyzer microplates containing the sensors cartridges and the drug delivery system to determine OCR;

12) Cultek, Madrid, ES, provided:

- Cultair BC 100 safety cabinet to tissue culture handling in aseptic conditions;

13) Thermo Scientific, Aalst, BE, provided:

- Microcentrifuge, model micro CL 17R, to centrifuge samples contained in eppendorfs;
- Steri-cycle CO_2 incubator Hepa class 100, model 371, to maintain tissue cultures at 37 ^{o}C with 5% (v/v) CO2 in air;

14) Hettich Zentrifugen, Madrid, ES, provided:

- Centrifuge, model Rotanta 460 R, to centrifuge samples contained in falcons;

15) MPW Med. Instruments, Warsaw, PL, provided:

- Centrifuge, model MPW-350R, to centrifuge samples contained in falcons;

16) Sheldon Manufacturing Inc., Cornelius, US:

- Water jacket CO_2 incubator, model 3517-2, to maintain tissue cultures at 37 ^{o}C with 5% (v/v) CO_2 in air;

17) Olympus, Lisbon, PT, provided:

- Digital camera integrated into the CKX41 microscope, model DP 20-5E, to take pictures to tissue cultures;
- Inverted microscope, model CKX41, to tissue culture visualization;

18) Leica Microsystems, Barcelona, ES, provided:

- Inverted microscope, model DM IL LED, to tissue culture visualization.

19) Clifton, provided:

- Thermoestatic water bath, model NE1B-14, to warm solution for tissue culture handling;

20) Hanna Instruments, Póvoa de Varzim, PT, provided:

- Benchtop pH meter, model HI 110, to adjust the pH of aqueous solutions;

21) Sartorius, Goettingen, DE, provided:

- Analytical balance, model ALC-810.2 (from the extinct brand Acculab), to prepare multiple solutions;
- Precision balance, model ED3202S, to prepare multiple solutions;

22) Mettler Toledo, Barcelona, ES, provided:

- Analytical balance, model AE 50, to prepare multiple solutions;

23) Eppendorf, Madrid, ES, provided:

- Adjustable-volume automatic pipettes, model Eppendorf Research with the references 3111 000.130, 3111 000.157 and 3111 000.165, to use in routinely laboratory tasks;

24) Gilson, Madrid, ES, provided:

- Adjustable-volume automatic pipettes, model PIPETMAN Classic with the references F144801, F144802, F123600, F123601 and F123602 to use in routinely laboratory tasks;

25) BMG Labtech, Madrid, ES, provided:

- Microplate reader, model FLUOstar OPTIMA, to detect luminescence;

26) BD Bioscience, Madrid, ES, provided:

- Flow cytometer, model BD FACSCalibur, to detect reactive oxygen species;