CREATION AND CHARACTERIZATION OF MITOCHONDRIAL DNA-DEPLETED HUMAN HUNTINGTON'S DISEASE AND CONTROL DERIVED LYMPHOBLASTS

Dissertação de Mestrado
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ABBREVIATIONS

ANOVA, One-way analysis of variance

ATP, adenosine triphosphate

bp, base pair

BDNF, brain derived neurotrophic factor

CAG, cytosine-adenine-guanine

CIs, cytoplasmic inclusions

CNS, central nervous system

CREB, cyclic-adenosine monophosphate response element binding protein

CoQ, coenzyme Q

Cx, complex

ddC, 2',3'dideoxycytidine

DTT, DL-dithiothreitol

ER, endoplasmatic reticulum

EtBr, ethidium bromide

ETC, electron transport chain

FBS, fetal bovine serum

GABA, γ–aminobutyric acid

GOF, Gain of function

HAP1, Htt-associated protein 1

HD, Huntington Disease

HEAT, Htt, elongation factor 3, the regulatory A subunit

HIP1, Htt-interacting protein 1

Htt, huntingtin

Hsp60, heat shock protein 60

IP3Rs, type 1 inositol (1, 4, 5)-trisphosphate

INIs, intracellular inclusions

IT15, Interesting Transcript 15

KI, knock-in

KO, knockout

LOF, Loss of function

mHtt, mutant huntingtin

MRC, mitochondrial respiratory chain

MRS, magnetic resonance spectroscopy

mtDNA, mitochondrial DNA

NADH, nicotinamide-adenine dinucleotide

nDNA, nuclear DNA

NMDAR, N-methyl-D-aspartate receptor

NR2, NMDA receptor subunit

NES, nuclear export signal

NLS, nuclear localization signal

NRSFs: neuron restrictive silence factor

OXPHOS, oxidative phosphorylation

PBS, phosphate buffered saline

PGC-1α, peroxisome proliferator-activated receptor γ coactivator 1-alpha

PMSF, phenylmethanesulfonyl fluoride

PET, positron emission tomography

POLG, polymerase gamma

PolyQ, polyglutamine (s)

PTP, permeability transition pore

PVDF, polyvinylidene difuoride

REST, repressor element 1 transcription

ROS, reactive oxygen species

rRNA, ribosomal RNA

tRNA, transfer RNA

SP1, specificity protein 1

SOD1, superoxide dismutase 1

TAFII, TATA-associated factors

TBS, Tris-buffered saline

TBP, TATA binding proteins

tRNA, transfer RNA

UPS, ubiquitin-proteasome system

YAC, yeast artificial chromosome

ΔΨm, membrane potential

ρ^0, rho zero

RESUMO

A doença de Huntington (HD) é uma doença neurodegenerativa autossómica dominante, caracterizada por perda neuronal selectiva dos neurónios do estriado e córtex. Os seus sintomas incluem movimentos corporais involuntários (nomeadamente coreia e distonia), alterações de personalidade, perda da habilidade cognitiva e demência. A sua causa deve-se à presença de expansões trinucleótidicas CAG no gene *IT15* ou *HD* que, neste caso, codifica uma proteína com um número de poliglutaminas, aumentado, a huntingtina mutante (mHtt). A mHtt interfere directa ou indirectamente em vários mecanismos inerentes à doença, incluindo a disfunção mitocondrial. Sabe-se que a presença de mHtt provoca um decréscimo na capacidade de tamponização do Ca^{2+}, no potencial de membrana ($\Delta\Psi m$), na actividade do complexo II, aumento de espécies reactivas de oxigénio (ROS), tráfego anormal de vesículas e alteração da dinâmica mitocondrial, o que culmina em morte neuronal. No entanto, desconhece-se a causa específica e a natureza da disfunção mitocondrial, associada à degeneração selectiva, presente na doença de Huntington. Nós últimos anos vários modelos, têm vindo a ser desenvolvidos, nomeadamente a criação de células rho zero (ρ^o), com intuito de avaliar a disfunção mitocondrial, assim como defeitos bioenergéticos característicos da doença de Huntington. O brometo etídeo (EtBr), é um agente que se intercala no DNA, que quando presente em concentrações baixas, inibe a replicação do mtDNA, sem afectar o nDNA, sendo, portanto, amplamente utilizado na obtenção de células ρo. Este trabalho teve como principal objectivo, criar e caracterizar células ρ^o a partir de linfoblastos controlo (CTR) ou doentes (HD). Neste trabalho as linhas de linfoblastos CTR ou HD foram incubadas com concentrações definidas de EtBr, 25 e 50 ng/ml durante 15 ou 30 dias, sendo posteriormente analisada a actividade dos complexos da cadeia respiratória mitocondrial, assim como a expressão de subunidades dos complexos I, II e IV. Os nossos resultados demonstram que a exposição a 25 e/ou 50 ng/ml de EtBr promove uma diminuição significativa na expressão da subunidade 20 kDa do Cx I e uma redução moderada na expressão da subunidade 57 kDa do Cx IV, ambas codificadas pelo mtDNA. Estas alterações são acompanhadas, pelo decréscimo da actividade do Cx I e do Cx IV. Em contraste, a expressão da subunidade 30 kDa do Cx I e da subunidade 70 kDa do Cx II ambas codificadas pelo nDNA, não são afectadas. No entanto, 60 dias após a remoção de EtBr, ocorre uma recuperação da

expressão das subunidades 20 kDa do Cx I e 57 kDa do Cx IV, ambas codificas pelo mtDNA, aumentam. Verificamos também que a expressão da Hsp60, uma proteína mitocondrial codificada pelo nDNA, permanece inalterada, após tratamento com EtBr, tanto em linfoblastos CTR como HD. Os resultados obtidos com este trabalho indicam que a exposição a concentrações definidas de EtBr inibe selectivamente a replicação do mtDNA, sem afectar o nDNA, fornecendo evidências para a possibilidade de criação de células ρo CTR e HD a partir de linfoblastos humanos.

Palavras Chave: Doença de Huntington; huntingtina mutante; disfunção mitocondrial; células ρ0; brometo de etídeo; linfoblastos; cadeia respiratória mitocondrial

ABSTRACT

Huntington disease (HD) is an autosomal dominant neurodegenerative disease characterized essentially by selective neuronal loss of neurons in the striatum. Symptoms include involuntary body movements (e.g chorea and dystonia), personality changes, loss of cognitive ability, leading to dementia. HD is caused by the presence of trinucleotide CAG expansion in *IT15* gene or *HD* gene, which encodes a protein with an increased number of polyglutamines, namely mutant huntingtin (mHtt). mHtt interferes directly or indirectly in various mechanisms of disease, including mitochondrial dysfunction. It is know that presence of mHtt cause a decrease in Ca^{2+} buffering capacity, decrease in mitochondrial membrane potential ($\Delta\Psi m$), defective bioenergetics, decrease of complex II activity, increase generation of reactive oxygen species (ROS), induce abnormal traffic of vesicles and impairment in mitochondrial dynamics, leading to neuronal death. However, the precise nature and cause underling mitochondrial dysfunction selective degeneration in HD is still unknown. In the last years, several models have been developed, including rho zero cells (ρ^o), to evaluation of mitochondrial dysfunction and bioenergetics defects that play an important role in HD. Ethidim bromide (EtBr) is the most frequent DNA intercalating agent used, which in low concentrations, inhibits mtDNA without affecting nDNA. In the present work CTR or HD lymphoblast cell lines were cultured in the presence of 25 and 50 ng/ml EtBr for 15 or 30 days, and further assayed for mitochondrial respiratory chain (MCR) complexes activities and associated subunits expression of complexes I, II and IV by western blotting analysis. However, 60 days after EtBr withdrawal, recovery in mtDNA-encoded Cx I 20 kDa and Cx IV 57 kDa subunits was observed. In addition we observed the expression of Hsp60. A mitochondrial protein encoded by nDNA, remains unchanged, that after treatment with EtBr. These data indicate that exposure to defined concentrations of EtBr, selectively inhibits the replication of mtDNA without affecting nDNA, providing evidence for the possible creation of ρ^o cells from CTR or HD human lymphoblasts.

Keywords: Huntington Disease; mutant huntingtin; mitochondrial dysfunction mitochondrial; ρ^0 cells; ethidium bromide; lymphoblasts; mitochondrial respiratory chain.

-CHAPTER 1. INTRODUCTION-

"I have drawn your attention to this form of chorea, gentleman, not that I considered it of any great practical importance to you, but merely as a medical curiosity, and as such it may have some interest". George Huntington, 1872"

1.1. HUNTINGTON´S DISEASE

Huntington´s disease (HD) was first described in 1872 by George Huntington, who identified both clinical features and pattern of familial transmission. HD is an autosomal, dominantly inherited neurodegenerative disease with a prevalence of 5-10 per 100,000 individuals in Europe and North of America (Gusella & Macdonald, 2006). The *HD* gene initially labeled *IT15* (*Interesting Transcript 15*) was identified for the first time in 1993 by a multicenter consortium, organized by the Hereditary Disease Foundation and a tremendous progress has been made since this discovery (Huntington´s Disease Collaborative Research Group, 1993). The molecular basis of the disease involves the expansion of the trinucleotide CAG (cytosine-adenine-guanine) in the first exon of the *HD* gene, located in chromosome 4 (4p16.3). The *HD* gene encodes for a widely expressed protein, huntingtin (Htt) with a molecular weight ~350 kDa, which in the mutant form (mutant huntingtin, mHtt) contains an elongated polyglutamine (polyQ) stretch in its N-terminal (The Huntington´s Disease Collaborative Research Group, 1993;(Gil & Rego, 2008). In the unaffected population, the number of CAG varies from 6-35 CAG units, while affected population have 36 or more CAG repeats (Gusella & Macdonald, 2007, Reddy *et al.*, 1999) (Fig. 1.1). The presence of 60 or more CAG repeats causes the juvenile-onset disease (Nance & Myers, 2001). In general, polyQ repeats are highly polymorphic and their length increase in every generation when expanded polyQ repeats are inherited through males, a phenomenon referred as genetic anticipation (Reddy *et al.*, 1999). There is an inverse correlation between the age of onset and the length of polyQ tract, whereby more CAG repeats are associated with an earlier disease onset (Fig. 1.1). However, the relationship between CAG repeat length and the age of onset differs when considering HD patients with juvenile onset or adult onset. The influence of each CAG appears to be stronger in the adult-onset range of CAG repeats than in juvenile-onset range (Andresen *et al.*, 2007). There are two chromosomal loci - one at 6q23–24 and the other at 18q22—that are capable of modifying the age of onset of HD. Interesting candidate

genes in these loci are serum and glucocorticoid regulated kinase gene (SGK) and metabotropic glutamate receptor gene (GRM1), for 6q23–24 and gene which encodes developmentally down-regulated 4-like gene (NEDD4L) for 18q(Li *et al.*, 2006)

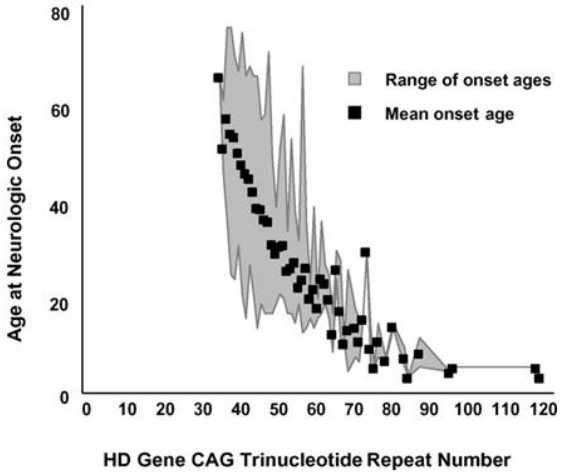


Figure 1.1: **The HD CAG trinucleotide repeat mutation and its relationship with age at neurological onset.** The mean age at neurological onset and the range of ages at onset associated with different HD expanded CAG repeats. Adapted from (Gusella & Macdonald, 2007).

HD typically manifests in mid-life, and terminates in death 10–20 years after the initial symptoms. Symptoms develop gradually over the disease progression and are categorized into 6 onset periods (Kirkwood *et al.*, 2001). Early symptoms vary from person to person but disease onset is generally marked by involuntary movements of the face, fingers, feet or thorax. Psychiatric symptoms are more heterogeneous but can occur before onset; they include depression, anxiety, apathy, and irritability (Duff *et al.*, 2007). The late stages are characterized by a variety of motor, emotional/behavioral, and cognitive symptoms, such as unsteadiness, trouble holding onto things, trouble walking, changes in sleeping patterns, hallucinations, intellectual decline, memory loss, difficulty in speech and weight loss. In the late stage patients lose bowel and bladder control (Kirkwood *et al.*, 2001).

The neuropathology markers involves the selective dysfunction and death of specific neuronal subpopulations within the central nervous system (CNS), namely GABAergic (γ–aminobutyric acid) projection medium/spiny neurons of the striatum (caudate and putamen), neurons in the cerebral cortex and, to lesser extent, in hippocampus (Spargo *et al.*, 1993, Vonsattel & Difiglia, 1998). However, with the disease progression, there is a general neuronal loss in several brain regions, such as globus pallidus, subthalamic nuclei, substantia nigra, cerebellum and the thalamus (Gil

& Rego, 2008, Spargo *et al.*, 1993). The extent of neuropathology and clinical symptoms was used to distinguish between 5 grades (0-4) of disease progression (Vonsattel *et al.*, 1985) (Fig. 1.2).

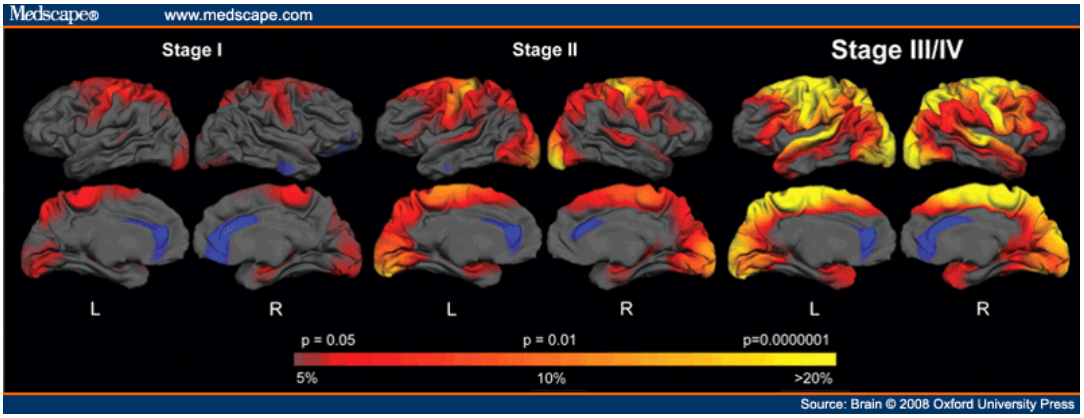


Figure 1.2. Model of disease progression. Selective dysfunction and death of striatal neurons and to lesser extent neurons within cerebral cortex. HD subjects were grouped according to stage. The colour scale at the bottom represent the thickness difference, with red to yellow indicating regions of more significant thinning in HD, compared to age matched controls. The magnitude of the brain thickness change is displayed as well, transitioning from red (5% loss) to yellow (>20% loss). Adapted from *http://www.medscape.com/viewarticle/573134_3*.

A characteristic feature of HD disease is the formation of intracellular aggregates, forming neuronal intranuclear inclusions (INIs) or cytoplasmic inclusions (CIs) in the affected brains (Ross & Poirier, 2005) (Fig. 1.3). The role of these protein aggregates remains controversial, since their formation is correlated with disease progression, but not associated with neuronal degeneration (Kuemmerle *et al.*, 1999). Thus, both protective (Arrasate *et al.*, 2004, Kuemmerle *et al.*, 1999) as well as toxic functions (Bates, 2003) have been described in the last years, for this aggregates.

In HD patients neurological symptoms predominate but, they are not the sole manifestations of the disease. Early reports described pathological phenotypes in peripheral tissues of HD patients, including weight loss, muscle wasting and altered glucose homeostasis (Sassone *et al.*, 2009). This suggests that cells from peripheral tissues of HD patients bear abnormalities related to expression of mHtt. Other reported changes included sub-cellular abnormalities in both fibroblasts and erythrocytes from HD patients (Sassone *et al.*, 2009) .

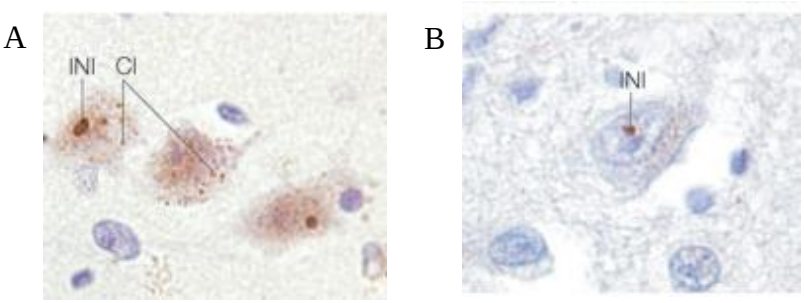


Figure 1.3 Intracellular aggregates in HD. A) Intranuclear inclusion and cytoplasmic inclusions B) Intranuclear inclusion. Visualized by light microscopy in the motor cortex of HD brain. Adapted from (Ross & Poirier, 2005).

1.2. HUNTINGTIN

Human Htt is a soluble large protein consisting of 3144 amino acids that has no similar sequence with other proteins. It has many potential domains whose boundaries and activities are not fully understood (Cattaneo *et al.*, 2001). The sequence is phylogenetically highly conserved, except the polymorphic proline-rich region adjacent to the polyglutamine tract (Faber *et al.*, 1998). Sequence analysis revealed that Htt contains multiple HEAT (Htt, elongation factor 3, the regulatory A subunit (PR65/A) of protein phosphatase 2A, and the lipid kinase TOR1) repeat sequences (Andrade & Bork, 1995), which are clustered into 4 major HEAT domains. Many phosphorylation and caspase-cleavable sites are located between the first two HEAT domains and present multiple targets for modulation and regulation of some events in HD pathogenesis (Warby *et al.*, 2009, Wellington *et al.*, 1998) (Fig. 1.4).

A functionally active C-terminal nuclear export signal (NES), sequence and nuclear localization signal (NLS) are present too. The NES defines a potential role of Htt as a member of nucleocytoplasmic dynamic protein complex, which may be important in HD because this fragment of protein is proteolytically cleaved in the disease (Xia *et al.*, 2003).

Various types of post-translational modifications may occur in Htt, including phosphorylation, ubiquitylation, SUMOylation, palmitoylation, transglutamination and proteolytic cleavage. The protein context and post-translational modifications influence Htt neurotoxicity (Pennuto *et al.*, 2009).

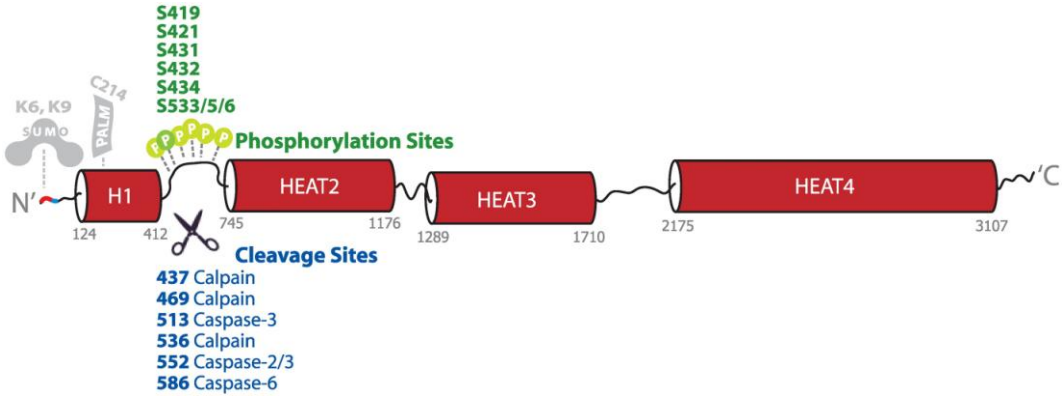


Figure 1.4. **Schematic diagram of Htt.** Htt is predominantly composed of HEAT repeats that comprise four major HEAT domains (red barrels). Several phosphorylation sites (green) are clustered in the intervening sequence between HEAT 1 and 2. Numerous cleavage sites (blue) are also found in this region. The location of characterized Htt SUMO and palmitoylation sites are also indicated. Adapted from (Warby *et al.*, 2009).

1.2.1. FUNCTION

The ubiquitous expression of Htt throughout the body, in neuronal and non-neuronal tissues, and its widespread localization at the subcellular level makes difficult to determine its function. It is not surprising that this protein is considered a scaffolding protein mediating protein–protein interactions playing a role in many cellular pathways (Macdonald, 2003). Several roles are assigned, depending on its subcellular localization and interaction with others proteins (Borrell-Pages *et al.*, 2006, Cattaneo *et al.*, 2005, Orr & Zoghbi, 2007). Consistent with this, it is known that Htt interact with a variety of proteins that can be grouped according to whether they are involved in gene transcription, intracellular signaling trafficking, endocytosis or metabolism (Harjes & Wanker, 2003, Li & Li, 2005).

Htt interacts directly with transcription factors, and might therefore act in the CNS as a general facilitator of neuronal transcription. Htt binds to the transcriptional repressor element 1 transcription/neuron restrictive silence factor (REST/NRSFs) in the cytoplasm, thereby preventing it from forming the nuclear co-repressor complex at RE1/NRSE nuclear site and allowing gene transcription (Zuccato *et al.*, 2003). Furthermore, *in vivo* data show that Htt stimulates cortical production of brain derived neurotrophic factor (BDNF), an important neurotrophin produced by projecting cortical neurons projecting to the striatum, necessary for the survival of striatal neurons (Zuccato *et al.*, 2001).

Another function that has been described for Htt is its involvement in intracellular trafficking. Htt was shown to be associated with proteins present in vesicle membranes (Difiglia *et al.*, 1995) and microtubules (Gauthier *et al.*, 2004), such as Htt-associated protein 1 (HAP1) and Htt-interacting protein 1 (HIP1) (Li & Li, 2005). Although both are associated with the trafficking, the HAP1 is associated with molecular motors dynein/dynactin (subunit $p150^{Glued}$), which is involved in microtubule dependent retrograde transport (Engelender *et al.*, 1997) and kinesin light chain, which is involved in anterograde transport 2 (Mcguire *et al.*, 2006). HIP1 is also important for assembly and function of the cytoskeleton, for endocytosis and binding of clathrin (a protein involved in the formation of coated vesicles) and alpha-adaptin subunit AP-2 (Waelter *et al.*, 2001). In addition, Htt directly promotes the microtubule-based transport of BDNF in neurons through this interaction (Gauthier *et al.*, 2004). Recently, studies *in vitro* and *in vivo* suggest that Htt may play a role in post-transcripcional transport/targeting of mRNA through association with neuronal RNA granules. These findings implicate a role of Htt in maintaining neurotrophic support and neuronal survival via delivery and processing of BDNF mRNA (Savas *et al.*, 2010).

Finally, Htt is an indispensable protein having anti-apoptotic properties, protecting neurons against apoptotic stimuli like serum deprivation, mitochondrial toxins or transfection of death genes (Cattaneo *et al.*, 2005, Rigamonti *et al.*, 2000). Htt acts downstream of mitochondrial cytochrome c release, preventing the formation of a functional apoptosome complex and the consequent activation of caspase-9 (Rigamonti *et al.*, 2001) and caspase 3 (Rigamonti *et al.*, 2000). In addition, Htt is essential for embryonic development and neurogenesis, as defined in different Htt knockout (KO)-mice, since complete inactivation of Htt in KO mice (Hdh -/-) causes embryonic death before day 8.5 (Nasir *et al.*, 1995, Zeitlin *et al.*, 1995). However, heterozygous KO mice appear either phenotypically normal (Duyao *et al.*, 1995) or display increased motor activity and cognitive deficits (Nasir *et al.*, 1995).

1.3. MECHANISMS OF HD PATHOLOGY: GAIN OR LOSS FUNCTION?

Since the discovery of the *HD* gene in 1993, a large number of studies on *post mortem* HD brain, cellular cultures and animal models revealed that a great number of cellular and, in particular, neuronal pathways and functions are abnormal in HD. However, the mechanism(s) responsible for triggering HD pathogenesis still remains

unknown. There are two hypotheses that explain the mechanism behind neuronal degeneration in HD: i) the gain of function (GOF) of mHtt and, ii) the loss of function (LOF) of normal Htt (Borrell-Pages *et al.*, 2006, Cattaneo *et al.*, 2001, Cattaneo *et al.*, 2005). According to the GOF hypothesis, the expanded polyQ causes a conformational change and confers a new function to Htt that is toxic to the cell (Fig. 1.5). This hypothesis is supported by the fact that patients with Wolf-Hirshhorn syndrome, a rare condition in which a deletion on chromosome 4 that comprises the CAG triplet repeats region (*HD* gene), occur do not develop HD (Gottfried *et al.*, 1981). This suggested that the presence of one fully functional allele is compatible with life in humans and that HD is not caused by a simple loss of function of *HD* gene. In contrast, in LOF hypothesis the decrease in expression of Htt due to interaction with mutant protein is thought to contribute to the disruption of intracellular homeostasis, culminating in neuronal dysfunction and death (Gil & Rego, 2008). As mentioned previously, Htt is an anti-apoptotic protein and promotes the transcription (Zuccato *et al.*, 2001) and microtubule-dependent transport (Gauthier *et al.*, 2004) of BNDF. Actually it is believed that HD pathology result of combined effect of GOF and LOF of normal Htt, leading to deregulation of relevant intracellular pathways that culminate in neurodegeneration and cell death.

1.3.1. WHEN HTT BECOMES TOXIC

In the last years there have been many attempts to determine the mechanisms by which the polyQ tract causes neurodegeneration in HD. It is known the pathogenic process begins with the synthesis of Htt with an expanded polyQ with a tract, altered native conformation that interferes with several pathways (Fig. 1.5). A fraction of abnormally folded protein is degradated by the proteasome, however expanded proteins are prone to misfolding and resistant to proteolysis by the ubiquitin-proteasome system (UPS), leading their accumulation within cell (Cummings & Zoghbi, 2000) and interference with proteosome function, leading to inhibition of proteosome activity (Venkatraman *et al.*, 2004). On the other hand, as described before, mHtt might undergo proteolytic cleavage by caspases, such as caspase 3, and also calpains, producing a toxic short N-terminal fragments that favours the aggregation process (Schilling *et al.*, 2006, Wellington *et al.*, 2000) (Fig. 1.4). This is supported by the fact that prevention of proteolysis by inhibiting caspases or calpain activation or by modifying the consensus cleavage sites in Htt reduces mHtt toxicity in *in vitro* and *in vivo* models (Gafni &

Ellerby, 2002, Wellington *et al.*, 2000). In addition, once cleaved, Htt fragments can translocate into the nucleus, where they have a greater affinity to bind to other proteins, such as nuclear proteins and transcription factors, forming aggregates, which affect transcriptional activity and may cause several deleterious events (Riley & Orr, 2006). Many proteins have been reported to interact with mHtt, such as TATA binding proteins (TBP), CREB binding proteins (CBP) (Schaffar *et al.*, 2004), specificity protein 1 (SP1) (Li *et al.*, 2002) and components of the basal machinery, such as essential subunits of RNAII complex, TBP, TFIIF, TAFII130 (Zhai *et al.*, 2005). For example one study demonstrated that the occupancy of RE-1/NRS loci by REST/NRSF is higher in HD, leading to decreased transcription of BDNF in HD cells, mice and humans *post mortem* samples, while inhibition of REST/NRSF binding restored BDNF levels (Zuccato & Cattaneo, 2007).

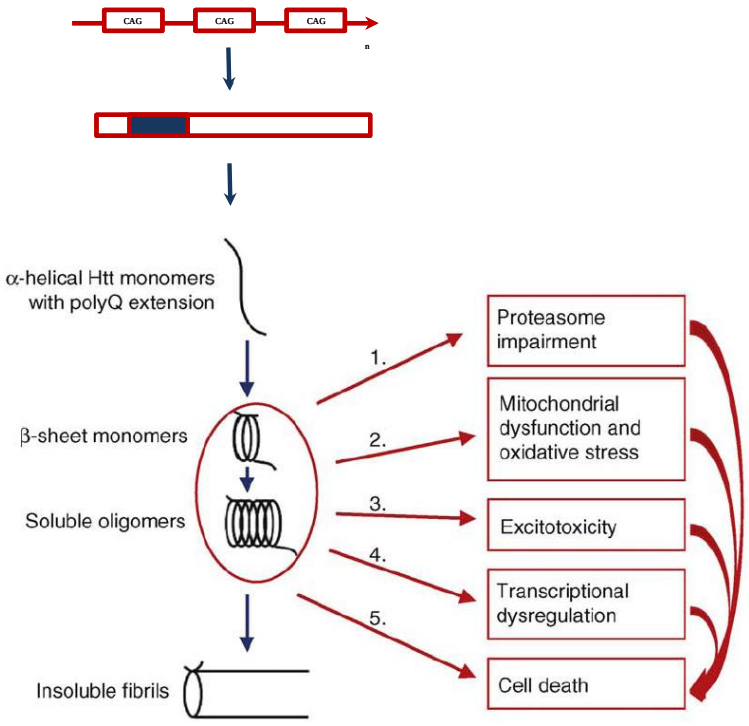


Figure 1.5. Model of pathogenic mechanisms in HD. Huntingtin proteins with >35 glutamine repeats fold into β-sheet structures. This might facilitates intermolecular cross-links by transglutaminases, leading to the accumulation of aggregates of misfolded Htt in the cytoplasm. Aggregates are toxic by a variety of mechanisms: 1. mHtt cannot be cleaved by the proteosome, leading to the accumulation of misfolded proteins 2. mHtt can interact with mitochondria causing dysfunction complexes of mitochondrial electron transport chain and decreased Ca^{2+} buffering (section 1.4.2.2), 3. Evidence from animal models and patients supports a role for excessive glutamatergic input (excitotoxicity) in HD pathogenesis but the molecular mechanism is not completely clear, 4. mHtt can be translocated into the nucleus and disrupt transcription, 5. mHtt can directly initiate pro-apoptotic signaling with activation of caspases and release of cytochrome c by mitochondria and cell death. However, all of these mechanisms may culminate in cell death. Adapted from (Fecke *et al.*, 2009).

As mentioned earlier, the presence of expanded protein in the cytoplasm can also interfere with microtubules BDNF. Indeed mHtt binds with high affinity to HAP1 and the p150 (Glued), causing an impaired association between motor proteins and microtubules leading to a reduced transport of BDNF vesicles along microtubules and loss of neurotrophic support (Gauthier *et al.*, 2004). In addition, mHtt (monomers and oligomers) can interact directly with mitochondria leading to caspases activation and mitochondrial dysfunction (Panov *et al.*, 2002).

Currently, there are two mechanisms that attempt to explain the formation of aggregates of mHtt: the polar zipper model (Perutz *et al.*, 1994) and the transglutamine model (Kahlem *et al.*, 1998). Polar zipper model refers the capacity that polyQ chains could theoretically form polar zippers, due to hydrogen bonds (Perutz *et al.*, 1994). In transglutamine model, transglutaminases to catalyze aggregation of Htt protein, especially in the expanded form (Kahlem *et al*., 1998).

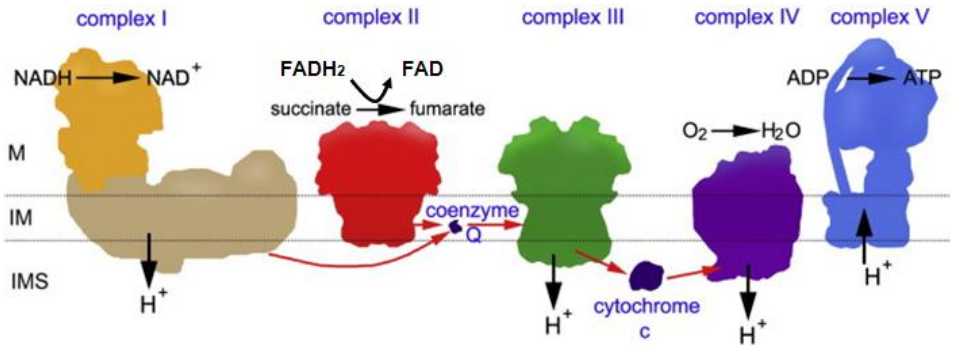
1.4. MITOCHONDRIAL DYSFUNCTION IN HD

1.4.1. MITOCHONDRIA

Mitochondria are highly dynamic organelles involved in multiple cellular processes of being ATP (adenosine triphosphate) production by oxidative phosphorylation (OXPHOS) the most prominent one (Schatz, 1995). However, mitochondria are also central to intracellular Ca^{2+} homeostasis (Celsi *et al.*, 2009), Krebs cycle and oxidation of fatty acids (Van Der Giezen & Tovar, 2005), generation of reactive oxygen species (ROS) (Benard *et al.*, 2007, Droge, 2002) and apoptotic pathways, involving proteins of Bcl-2 family of proteins and the release of cytochrome c and other pro-apoptotic factors (Spierings *et al.*, 2005).

The human mitochondrial DNA (mtDNA) genome is small 16,569 base pair (bp) in a long circular chromosome composed of double-stranded DNA, and 37 genes which encode for RNA components of the mitochondrial translation apparatus, 22 transfer RNA (tRNAs) genes and 12S and 16S ribosomal RNA (rRNA) genes, as well as 13 polypeptide-encoding genes (mRNAs). All 13 polypeptides are essential components of four of the five complexes that form the mitochondria OXPHOS complex (Cx) (Attardi & Schatz, 1988). Seven polypeptides, ND1, ND2, ND3, ND4, ND4L, ND5, ND6, are subunits of Cx I (NADH-dehydrogenase-ubiquinone reductase); cytochrome b is part of Cx III (ubiquinol-cytochrome c reductase); COI, COII and COIII are catalytic subunits

of Cx IV (cytochrome c oxidase), and ATPase 6 and 8 are subunits of Cx V. All the subunits of Cx II (succinate dehydrogenase-ubiquinone reductase) are encoded by nuclear DNA (nDNA) (Fig. 1.6) (Zeviani & Antozzi, 1997). Therefore, each Cx of the mitochondrial respiratory chain (MRC) (except Cx II) contains subunits encoded by nuclear genes, which are assembled together with the mtDNA-encoded subunits into the respective haloenzymes, located in the inner mitochondrial membrane (Scarpulla, 1997). Human mtDNA lacks the protection by histones, DNA binding proteins, and is replicated without efficient proofreading and a DNA repair system. Moreover, mtDNA is highly exposed to ROS that are continually generated by the MRC and to other free radicals. The random hit of the naked mtDNA by ROS or free radicals is likely to cause oxidative damage or mutations (Wang *et al.*, 2003). Accumulation of mutations and oxidative damage to mtDNA result in MRC dysfunction, leading to increased production of ROS in mitochondria and induction of further mtDNA mutations. mtDNA damage due to oxidative stress has been observed in cortex of *pos tmortem* HD brains (Polidori *et al.*, 1999), and in animal models such as transgenic mice R6/2 (Acevedo-Torres *et al.*, 2009).



Subunits	Complex I	Complex II	Complex III	Complex IV	Complex V
mtDNA	7	0	1	3	2
nDNA	36	4	10	10	14

Figure 1.6. **Energy production through the coupling of MRC with OXPHOS in the mitochondria.** The reducing equivalents in NADH or $FADH_2$ enter the electron transport chain thought Cx I and Cx II, respectively. During the transfer of electrons from NADH to coenzyme Q (CoQ), from CoQ to Cx III, and then from cytochrome c to Cx IV, protons are translocated from the matrix to the intermembrane space. A proton gradient is thus established across the mitochondrial membranes, which is the driving force for ATP synthesis catalyzed by membrane-located ATP synthase (Cx V). The subunits of complexes are encoded by two genetic system: the nDNA and mtDNA. Adapted from (Dudkina *et al.*, 2010).

1.4.2. POSSIBLE MECHANISMS OF MITOCHONDRIAL DYSFUNCTION

Mitochondria are organelles of great importance in the cell, being mitochondrial dysfunction a hallmark of many common neurodegenerative diseases (Beal, 2005, Knott *et al.*, 2008). Evidences of mitochondrial dysfunction associated to the pathogenesis of HD have been accumulated over the last 30 years (Section 1.4.2.1.). Many HD models have been generated using mitochondrial toxins (Brouillet *et al.*, 2005), which stemmed from *post mortem* brain data (Gu *et al.*, 1996).

Several different models are available for HD research as represented in Table I. Thus, it is unquestionable the involvement of mitochondrial dysfunction in the process of disease, but its precise nature and cause remain uncertain (Browne & Beal, 2004, Oliveira, 2010).

It is known that mHtt interacts directly or indirectly with mitochondria, interfering with mitochondrial function, including a reduction of Ca^{2+} buffering capacity, loss of mitochondrial membrane potential ($\Delta\Psi m$), impairment in MRC complexes, increased generation of ROS, abnormal vesicle trafficking and impairment

in mitochondrial dynamics, leading to neuronal death. The role of mitochondrial dysfunction in HD has been recently reviewed (Bossy-Wetzel *et al.*, 2008, Browne, 2008, Pandey *et al.*, 2010, Reddy *et al.*, 2009).

Table I. Different models used in the study of mitochondrial dysfunction in HD.

Model	Form of huntingtin
• Cell line (s)	• Full length of mhtt, polyQ only, exon1 containing polyQ repeats
• Striatal cell culture	• Full length of mHtt
• **Lymphoblasts**	• **Wild type and mHtt**
• Yeast (*Saccharomyces cerevisiae*)	• mHtt
• Nematode (*Caenorhabditis elegans*)	• Exon 1 of human htt
• Fruit fly (*Drosophila sps*)	• N terminal of mHtt
• R6 transgenic line in mice	• N terminal of exon 1 human htt, Larger N terminal (N171-82Q)
• HD line in mice	• 1 kb of human Htt full length of human htt
• YAC* model in mice	• Full length human Htt
• Knock in mouse	• Mutated mouse Htt, Htt exon 1 of mouse replaced by mHtt exon 1 of human
• Excitotoxin models in rats: Kainic acid, Quinolinic acid,	• ----------
• Mitochondria complex-II inhibitors in rats: malonate, 3-nitropropionic acid.	• ----------
• Mitochondrial complex-II inhibitors in Baboons (*Papio anubis*)	• ----------
• Transgenic model in monkey (*Macaca sps*)	• Human mHtt exon 1

Different models and correspondent form of mhtt.

*Yeast artificial chromosome

Adapted from (Pandey *et a*l., 2010).

1.4.2.1. Respiratory chain impairment

Since early biochemical studies, defects in enzymes of oxidative metabolism have been observed in HD, and thus respiratory chain impairment was proposed as a primary triggering event in HD pathogenesis. Despite controversial results obtained in different models, it is nowadays believed that mitochondrial impairment is a secondary event in the pathogenesis of HD (Oliveira, 2010).

Energetic impairment in HD patients has been observed by a variety of methods, such as, Positron Emission Tomography (PET) that showed a significant reduction in glucose uptake in cortex and striatum of HD patients (Gil & Rego, 2008). Through the use of Magnetic Resonance Spectroscopy (MRS), it was found that levels of lactate are diminished in cortex of symptomatic patients and in striatum of presymptomatic HD patients (Jenkins *et al.*, 1998). Impairment in MRC enzyme activities has been demonstrated such as, activity of succinate dehydrogenase, Cx II, cytochrome oxidase, Cx IV (Brennan et al., 1985), pyruvate dehydrogenase (Butterworth *et al.*, 1985) and aconitase (Sorolla *et al.*, 2008, Tabrizi *et al.*, 1999) in striatum of HD patients.

Post mortem samples of patients with striatum atrophy revealed reduced activity in complexes II/III and a mild reduction in Cx IV (Browne, 2008, Gu *et al.*, 1996). Accordingly, expression of two subunits of Cx II (Ip and Fp) are preferentially decreased in the striatum of HD patients compared with controls (CTR) subjects and these alteration affect the dehydrogenase activity of the Cx (Benchoua *et al.*, 2006). Still, in these studies, the activity of Cx I was not altered. Nevertheless, deficits in Cx I have been described in different peripheral tissues from HD patients, such as platelets (Parker *et al.*, 1990) and muscle (Arenas *et al.*, 1998). However, several different studies could not reproduce such deficit in *post mortem* HD brain or in HD patient's platelets (Gu *et al.*, 1996, Powers *et al.*, 2007), muscles (Turner *et al.*, 2007) and lymphoblasts (Sawa *et al.*, 1999). In genetic HD models, particularly mice expressing full-length mHtt, no significant alterations were found in measurements of MRC Cx I-IV in striatum and cerebral cortex (Browne & Beal, 2004, Guidetti *et al.*, 2001). It should be noted that in digitonin-permeabilized striatal cell lines that exhibited a significant decrease in MRC rates, no impairment in individual respiratory complexes was detected (Milakovic & Johnson, 2005). The same authors observed that differences in MRC rates disappeared when using isolated mitochondria from the same cell lines

(Milakovic *et al.*, 2006), suggesting that detection of mitochondrial deficits requires a preserved cellular context.

1.4.2.2. Mitochondrial Ca^{2+} buffering capacity in HD

Mitochondria are involved in the maintenance of Ca^{2+} homeostasis mainly because of their capacity to buffer cytosolic Ca^{2+} (Budd & Nicholls, 1996). Several lines of evidences suggested that abnormal Ca^{2+} uptake occurs in HD neurons (Reddy *et al.*, 2009) (Fig. 1.7). It is believed that mHtt interacts directly with mitochondrial membranes causing deleterious effects. A decreased mitochondrial Ca^{2+} loading capacity and lower ΔΨm were observed in lymphoblasts from HD patients (Panov *et al.*, 2002). This vulnerability was proportional to mHtt levels (Panov *et al.*, 2002). Similar defects were observed in brain mitochondria from transgenic yeast artificial chromosome (YAC) mice expressing full-length mHtt (Panov *et al.*, 2002). Subsequent studies, using knock-in (KI) HD mice (50Q, 92Q, 111Q and 150 Q) revealed either no differences or decreased susceptibility to Ca^{2+} loads, expressed as increased Ca^{2+} buffering capacity (Bezprozvanny & Hayden, 2004). The same was observed in brain mitochondria isolated from diverse HD mice such as R6/2, revealing either no differences or decreased susceptibility to Ca^{2+} loads, expressed as increased Ca^{2+} buffering capacity (Brustovetsky *et al.*, 2005, Oliveira *et al.*, 2007). However, more recently brain mitochondria from HD rats exhibited diminished ΔΨm stability in response to Ca^{2+}, lower capacities and rate of Ca^{2+} accumulation when compared to CTR rats (Gellerich *et al.*, 2008). One explanation for these results is that the buffering capacity may change between different models and isolated mitochondria may be influence methodological approaches (Oliveira *et al.*, 2007).

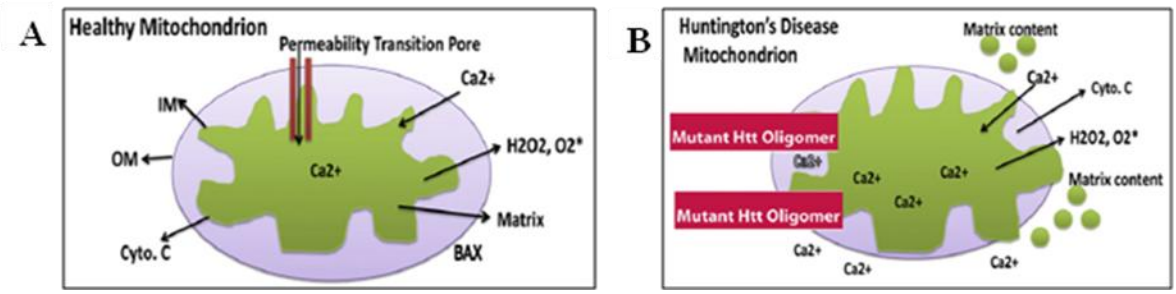


Figure 1.7.**Mitochondrial Ca^{2+} buffering capacity.** A) In healthy mitochondria, the inner mitochondrial membrane provides a highly efficient barrier to ionic flow and protects mitochondria from toxic insults B) Excess mitochondrial Ca^{2+} load may open the mitochondrial permeability transition pore in mitochondria from HD patients, leading to cytochrome c release. Adapted from (Reddy *et al.*, 2009).

Therefore these studies suggest that ΔΨm and mitochondrial Ca^{2+} regulation are directly impaired by mHtt and that increased ROS generation may be driving these alterations (Panov *et al.*, 2002, Puranam *et al.*, 2006).

Cytosolic Ca^{2+} can also be mobilized by the endoplasmic reticulum (ER), which is another major store for intracellular Ca^{2+}. mHtt forms a ternary complex with Huntingtin associated protein 1 (HAP-1A) and type 1 inositol 1,4,5-trisphosphate (IP_3Rs). In this complex, mHtt facilitates the release of Ca^{2+} from the ER and renders neurons more sensitive to Ca^{2+} mediated cellular dysfunction (Bezprozvanny & Hayden, 2004). Additionally, mHtt enhances Ca^{2+} entry through the *N*-methyl-D-aspartate (NMDA) receptors, leading to Ca^{2+} desregulation and consequent activation of caspases and calpain, leading to cell death (Bezprozvanny & Hayden, 2004). Indeed, several approaches have shown that mHtt can directly modify NMDA-receptor function through its interaction with PSD-95 (Song *et al.*, 2003, Sun *et al.*, 2001). In particular, mHtt increases the sensibility of neurons to excitotoxicity associated to the stimulation of NMDA receptors contain the NR2B subunits (Zeron *et al.*, 2002).

According to Choo et al. (2004), mHtt induces mitochondrial permeability transition pore (PTP) (Fig. 1.7) in isolated mouse liver mitochondria. Moreover from studies in mouse striatal neurons, where authors observed the opening of PTP in permeabilized polyQ expressing cells (Lim *et al.*, 2008). Therefore, the PTP appears to be a final commitment step in a number of cellular stress conditions, with Ca^{2+} acting as a potent sensitizing factor (Lim *et al.*, 2008).

1.4.2.3 Mitochondrial trafficking deficits and dynamics

Mitochondrial trafficking deficits are a recent proposed mechanism for mitochondrial dysfunction in HD. The first study was conducted by Trushina and co-workers (2004) who analysed the impact of mHtt on mitochondrial transport. Accordingly, mHtt impair movement indirectly by the sequestration of machinery components and Htt, which is essential for axonal transport (Trushina *et al.*, 2004) or by physical blockage of axonal transport (Chang *et al.*, 2006).

Accordingly, Chang et al. (2006) reported that mHtt aggregates act as physical roadblocks for mitochondrial transport in cortical neurons; consequently, in the narrow neuronal projections these aggregates prevent passage of mitochondria and fragmented mitochondria accumulate around mHtt. They proposed that this impairment in mitochondrial movement was an early pathogenic event, occurring before mitochondrial

and cellular dysfunction in cortical neurons (Chang *et al.*, 2006). In addition, it was also reported that mHtt induces changes in mitochondrial morphology from elongated to a round phenotype (Chang *et al.*, 2006). More recently, another study demonstrated that mHtt associates with microtubule based transport proteins decreasing mitochondrial transport in striatal neurons (Orr *et al.*, 2008). This mechanism may be behind the vulnerability of striatal neurons, in HD disease.

Therefore, mHtt change the mitochondrial trafficking by several ways; in other, mHtt aggregates may block mitochondrial movement or/and mHtt may heavily interact with trafficking proteins, which may block/derail mitochondrial movement in the axon (Reddy *et al.*, 2009). In addition, a large number of defective mitochondria accumulate due to excessive mitochondrial fragmentation in HD neurons; mHtt may create an imbalance between mitochondrial fission and fusion, leading to decrease in overall mitochondria dynamics in neurons. All these events may be responsible for low ATP production, mitochondrial dysfunction, and damaged medium spiny neurons in HD (Reddy *et al.*, 2009).

1.4.2.4 Transcriptional dysregulation in HD

As previously described, mHtt induces transcriptional deregulation via interference with transcriptional factors occupation of genes promotes, and even direct DNA binding (Benn *et al.*, 2008, Zhai *et al.*, 2005). Because p53 regulates many apoptotic mitochondrial (Bax and Puma) and oxidative stress responsible genes (Vogelstein *et al.*, 2000), the strong interaction between mHtt and p53 accumulation in the nucleus and thus induction of p53-dependent transcription (Bae *et al.*, 2005) (Fig. 1.7). However, in intracellular polyQ aggregates p53 soluble levels are decrease in HD. (Suhr et al., 2001).

Another very interesting mechanism that has recently been proposed is the peroxisome proliferator-actived receptor γ coactivador-1α (PGC-1α) pathway, an important orcheastrator of mitochondrial function via integration of signals that regulate mitochondrial respiration, oxidative stress defense and adaptive thermogenesis (Cui *et al.*, 2006, Puigserver & Spiegelman, 2003, St-Pierre *et al.*, 2006). PGC-1α promoter has been shown to associate with mHtt, and interfere with CREB/TAF4 dependent transcriptional pathway, which is important for PGC-1α expression (Cui *et al.*, 2006). mHhtt induces PGC-1α transcriptional repression, which is associated with mitochondrial dysfunction and neurodegeneration. A very recent study reports that

PGC-1α levels are reduced in muscles of HD patients and in transgenic HD mice NLS N171-82Q (containing 82 polyQ repeats), as compared to wild type littermates, confirming the active involvement of this transcriptional co-activador in HD pathology (Chaturvedi *et al.*, 2009). It was found that PGC-1α decreased in HD *post mortem* brains, in cell lines expressing mHtt, and in HD mouse models, suggesting that mHtt promotes the increased production of ROS due to an increase in PGC-1α. Related with this increase in PGC-1α is an increase in scavenging enzymes such as superoxide dismutase (SOD)1 (Cu/Zn-SOD) or 2 (Mn-SOD), catalase and glutathione peroxidase (Arany *et al.*, 2008, Cui *et al.*, 2006, St-Pierre *et al.*, 2006, Weydt *et al.*, 2006). Although the findings of transcriptional dysregulation by mHtt are of high relevance, they can not entirely explain all the mitochondrial defects observed in HD. Therefore, more studies will be necessary to better understand this and other mechanisms.

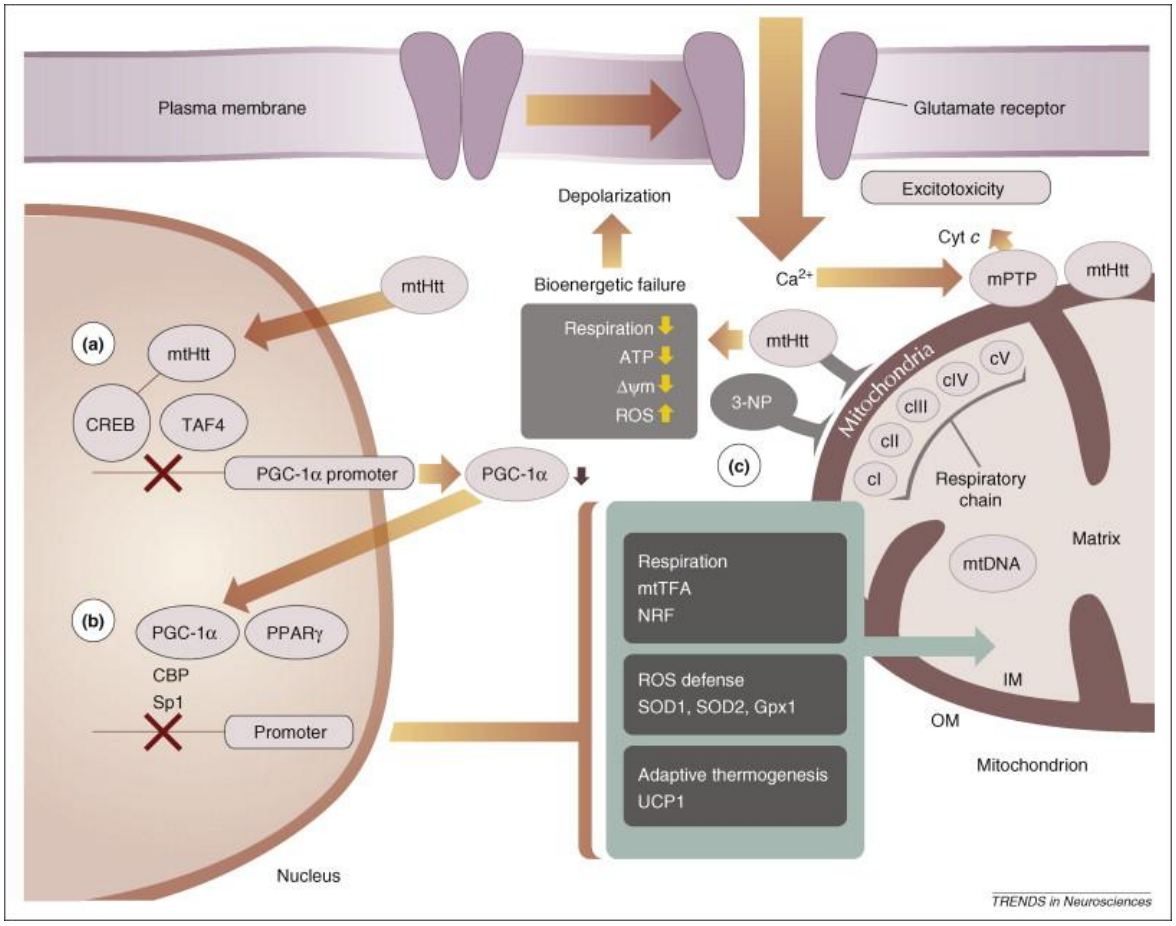


Figure 1.8. mHtt and mitochondrial dysfunction in HD. Representative direct and indirect mechanisms involving mHtt and mitochondria. A) mHtt blocks the PGC-1α promoter via inhibition of the CREB transcriptional activator, resulting in decreased PGC-1α expression. B) Lowered PGC-1α decrease PPARγ-mediated expression of nuclear-encoded mitochondrial proteins that are necessary for respiration and oxidative-damage defense. C) Direct interaction with mitochondria blocks the respiratory complex II, similarly to 3-nitropropionic acid (3-NP). Respiratory chain, in turn, leads to decreased energy production and decreased ΔΨm in addition to increased ROS generation. The bioenergetic declines caused by transcriptional deregulation and direct effects on mitochondria can cause increased vulnerability to excitotoxic stimuli and amplification of the mitochondria damage Ca^{2+}-mediated PTP opening. Adapted from (Bossy-Wetzel *et al.*, 2008).

1.5. RHO ZERO AS A MODEL FOR STUDYING MITOCHONDRIAL DYSFUNCTION

Several mtDNA depleted mammalian cells lines have been generated to investigate the role of mitochondria in aging and age related disorders , such as aging and age-related, namely Alzheimer's and Parkinson's disease (Chomyn *et al.*, 1994, Marusich *et al.*, 1997, Miller *et al.*, 1996). The development of cells depleted of mtDNA has provided a suitable model to study some of the molecular mechanisms governing mitochondrial defects. Such cells, defined as rho-zero (ρ^0) have been produced by long-term culture with compounds such as ethidium bromide (EtBr) a cationic and lipophilic agent that damage and inhibits mtDNA replication and transcription when present under lower concentration (King & Attardi, 1989). Additionally, other methods were tested to generated ρ^0 cells, such as dideoxynucleoside analogs (i.e 2',3'dideoxycytidine (ddC)) (Nelson *et al.*, 1997), a antiviral nucleoside analog that inhibits mtDNA replication (Martin *et al.*, 1994), exposure to rhodamine 6-G, a lipophilic dye which degrades mammalian mitochondria (Trounce & Wallace, 1996) and more recently based on an enzymatic approach (Kukat *et al.*, 2008). This method destroy endogenous mtDNA *in vivo* was also based on a restriction endonucleases that was target to the matrix of mitochondrial thereby cleaving the genome and allowing endogenous enzymes to fully disintegrate the DNA molecules (Kukat *et al.*, 2008). EtBr inhibits mtDNA polymerase gamma (POLG) more strongly than DNA polymerase alpha and beta, thus inhibiting the replication and transcription of mtDNA without substantially affecting nDNA (Qian & Van Houten, 2010).

Rho-zero cells are devoided of mtDNA and electron transport chain (ETC) activity and are dependent on uridine and pyruvate, for growth because of the absence of a functional respiratory chain (Desjardins *et al.*, 1985, King & Attardi, 1989) i. e, these cells present inability to synthesize a particular compound (in this case uridine and pyruvate) required for its growth (auxotrophic). The growth medium needs supplementation with nutrients to sustain viability. This is achieved by adding pyruvate (to regenerate NAD^+ following its conversion to NADH (nicotinamide adenine dinucleotide) in glycolysis and thus anaerobic ATP generation) and uridine (to facilitate pyrimidine synthesis, which becomes ineffective under conditions of ETC failure), in order to prevent energy demand of cell is satisfied (King & Attardi, 1989, Miller *et al.*,

1996, Swerdlow *et al.*, 1997, Swerdlow *et al.*, 1999). After EtBr treatment, these authors observed a reduction in mtDNA amount. However, if the DNA-intercalating agent is removed before complete depletion of mtDNA, cells repopulate with residual genomes in a period that will depend on the size of the mtDNA (Moraes *et al.*, 1999). Cells with large deletions, but not with pathogenic point mutations, repopulate organelles faster than wild-type genomes in the same cell, particularly during relaxed copy number control (Diaz *et al.*, 2002)

1.5.1. CYBRIDS

One of the applications for the creation of ρ^0 cells is the ability to create cytoplasmic hybrids (cybrids), first described in mammals by King and Attardi, in 1989 as a new model of disease for mitocondrial researches. In general, the cybrids are created when cytoplasmic contents of two different cells are processed within a single plasma membrane.This presents distinct applications in the study of mitochondrial function, including the study of mutations in mtDNA, assessing the integrity of the transferred mtDNA or compatibility biogenomic (Khan *et al.*, 2007, Swerdlow, 2007) and more recently as a platform for the development of new therapies (Trimmer & Bennett, 2009).

Despite the various approaches to create cybrid lines, the most commonly used technique involves the transfer of mitochondria from non-nucleated cells (usually platelets) to ρ^0 cells, resulting in cybrids containing nDNA from ρ^0 cells and mtDNA from patient's or donor's platelets (Chomyn *et al.*, 1994) (Fig. 1.9). After fusion, host cells repopulated with platelet-derived mitochondria undergo metabolic selection to eliminate cells with incomplete repopulation (Swerdlow *et al.*, 1997).

Presently this is a technique widely used in the study of many neurodegenerative diseases, such as Parkinson´s disease (Esteves *et al.*, 2008, Trimmer & Bennett, 2009), Alzheimer's disease (Cardoso *et al.*, 2004, Swerdlow *et al.*, 1997) and Huntington's disease (Ferreira *et al.*, 2010, Swerdlow *et al.*, 1999).

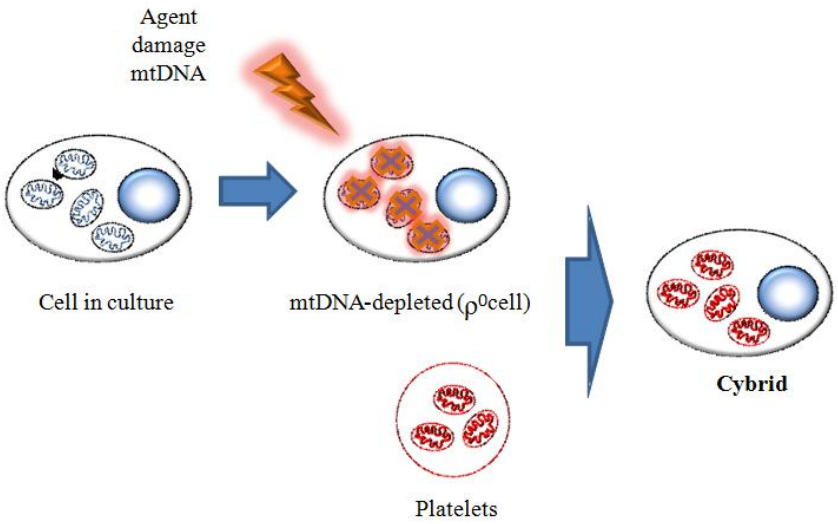


Figure 1.9. Cybrids technique. ρ^0 cell produced by long term culture with compounds that damage mtDNA. Transfer of mitochondria from platelets to ρ^0 cells, resulting in hybrids cells (cybrids) containing nDNA from ρ^0 cells and mtDNA from donor platelets.

1.6. OBJECTIVE

Despite major research efforts on HD, the underlying mechanisms leading to selective degeneration of striatal neurons in HD are still largely unknown and no therapy is currently available for this fatal disease. Several mechanisms of mHtt toxicity have been proposed, which partially fit with clinical data gathered from HD patients as well as from molecular, cellular and animal experiments. Currently, it is not known if one of these different mechanisms previously described triggers the other or if these different mechanisms, involved in many pathways, could participate synergistically in the pathology. It is well accepted that mHtt is widely expressed not only in the brain but also in peripheral tissues, suggesting that an adverse effect of mHtt is not limited to neurons (Rosenstock *et al.*, 2010, Sassone *et al.*, 2009). Several reports described alteration in peripheral tissues of HD patients, including platelets (Parker *et al.*, 1990), lymphocytes (Almeida *et al.*, 2008, Sawa *et al.*, 1999) and muscles (Arenas *et al.*, 1998, Turner *et al.*, 2007).

Human HD lymphoblasts have been used in many studies as a cellular model of HD in mitochondrial dysfunction (Sassone *et al.*, 2009). Many alterations present in HD neurons are present in lymphoblasts from HD patients, namely decreased $\Delta\Psi m$ (Panov *et al.*, 2002), impaired Ca^{2+} buffering (Panov *et al.*, 2002), mitochondrial morphological alterations (Squitieri *et al.*, 2006) and the presence of genetic instability (Cannella *et al.*, 2009, Squitieri *et al.*, 2006).

To gain further insight into the pathology, the main aim of this thesis was to create and characterize a new human cell model of mitochondrial dysfunction in HD, namely HD *versus* control (CTR) ρ^0 lymphoblast cells. For this purpose, different lymphoblast cell lines were cultured in the presence of 25 and 50 ng/ml and EtBr for 15 or 30 days. This model was further characterized by analyzing several proteins encoded by mtDNA and nDNA, namely subunits of complexes I, II and IV, and the activity of MRC complexes I and IV.

This model will allow a better understanding of the role of mitochondria in HD, as well as the effects of mitochondrial dysfunction in this devastating neurodegenerative disease.

-CHAPTER 2. MATERIAL & METHODS-

2.1. MATERIAL

Lymphoblastic cell lines were obtained from NIGMS Human Genetic Cell Repository (CORIELL Institute for Medical Research, New Jersey, and USA). RPMI-1640 medium, phenylmethanesulfonyl fluoride (PMSF), DL-Dithiothreitol (DTT), protease inhibitor cocktail, FBS, EtBr, uridine and pyruvate were obtained from Sigma Chemical Co, St Louis, MO, USA. Antibodies against-Cx I 20 kDa subunit and Cx IV 57 subunit were obtained from Invitrogen (Carlsbad, USA). Cx I 30 kDa and Cx II 70 kDa subunit were obtained from Molecular Probes, Leiden, Netherlands and antibody against heat shock protein (Hsp60) was obtained from Chemicon, Hampshire, UK. ChemiFluorescence reagent ECF and anti-mouse secondary antibody were obtained from GE Healthcare (Little Chalfort, UK). All other reagents were of analytical grade.

2.2. LYMPHOBLASTOID CELL LINES

Lymphoblast cell lines were produced by CORIELL Institute. Accordingly to data sheet, lymphocytes were obtained from peripheral blood of HD patients or control (CTR) subjects, cultured by using phytohemagluttinin as a mitogen and then infected by Epstein-Barr virus in order to obtain lymphoblastoid cell lines. In this study, lymphoblast cell lines were obtained from HD affected patients containing heterozygous expansion mutation (n=4, three males (43/15, 45/15, 42/18) and one female (47/18) (Table II), or from unaffected aged matched voluntary subjects (control siblings) (n=2, one male and one female), used in this work as CTR lymphoblasts (Table III). Human peripheral blood was obtained after informed consent.

Table II. Demographic and genetic characteristics of HD lymphoblast cell lines.

Cell line	Gender	Age at onset	Age at Sampling	Expanded CAG	Race
#4798	Male	42 yrs	47 yrs	43/15	Caucasian
#5610	Female	40 yrs	52 yrs	47/18	Caucasian
#5622	Male	38 yrs	41 yrs	45/15	Caucasian
#5678	Male	48 yrs	58 yrs	42/18	Caucasian

Catalog number of caucasian HD lymphoblasts and detailed information about gender, age of disease onset, age of sampling and respective number of CAG repeats.

Table III. Demographic characteristics of the CTR lymphoblast cell lines.

Cell line	Gender	Age at onset	Age at Sampling	Expanded CAG	Race
# 4800	Female	-----	45 yrs	-----	Caucasian
#4808	Male	-----	42 yrs	-----	Caucasian

Catalog number of caucasian CTR lymphoblasts and detailed information about gender and age of sampling.

2.3. CELL CULTURE

Lymphoblast cells were shipped in T25 tissue culture flasks that have been filled to capacity with carbon dioxide-equilibrated medium to provide sufficient nutrients for extended transport times. Upon receipt, flasks containing lymphoblasts were incubated unopened overnight at 37°C in upright position, with vented or loose caps. Lymphoblast cultures were counted in the next day and split if sufficient growth has occurred. Alternatively, the volume of the culture medium was decreased to yield a cell density of 200,000 - 500,000 viable cells/ml. Lymphoblasts were then cultured in RPMI 1640 medium supplemented with 15% of non-inactivated FBS plus 2 mM glutamine and 50 µg/ml streptomycin plus 100 IU/ml penicillin in T25 or T75 flasks, in upright position, by using an incubator chamber containing 5% CO2, 95% air, 100% humidity at 37ºC. In these conditions, lymphoblastoid cell lines grew in suspension with cells clumped in loose aggregates (Fig. 2.1 A and B). When desired, these aggregates were dissociated by gently agitating the culture or by gentle trituration with a pipette. In three to four days, the culture was either re-fed with fresh medium or split again taking into account

how fast the particular line grows, or the desired number of cells needed for the experiments.

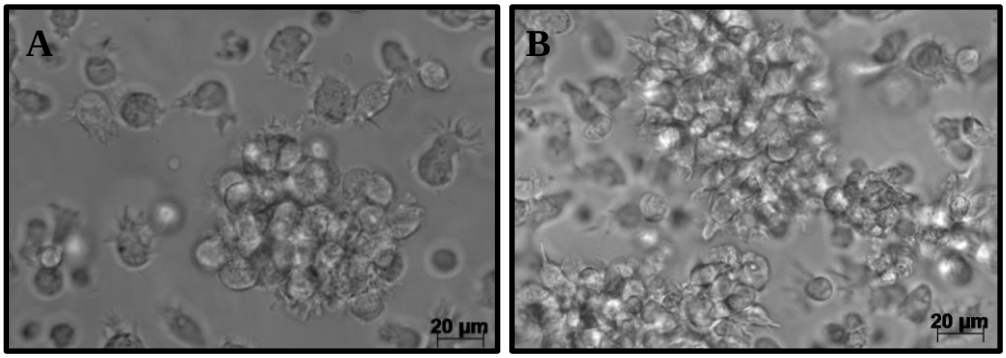


Figure 2.1. Morphological features of lymphoblast cell lines. Representative images of aggregates of CTR (A) or HD (B) lymphoblast cell lines in culture, visualized by PALM MicroBeam inverted microscope.

2.4. PROLIFERATION CURVES

Trypan blue test is commonly used to evaluate cell viability in a cell suspension It is based on the principle that live cells having intact plasma membranes exclude the dye, whereas dead cells do not. In order to study the rate of division of the lymphoblast cell lines, cells were seeded at a density of $0.2x10^6$ cell/ml in 48-well plates and cultured for 5 days. Every other day, an aliquot of cell suspension was two times diluted in 0.1 % trypan blue and counted by using a hemocytometer under inverted light microscopy (Fig. 2.2 A-E). The plateau level for most cultures was reached at about 1×10^6 viable cells/ml three to five days after sub-culturing. The pH of cultures was shown to be quite acidic, appearing distinctly yellow at this point since phenol red was used in the culture medium as pH indicator. Cultures left in the plateau phase exhibited a decrease in viability accompanied by a lengthening of the doubling time. All our experiments were performed in cultures presenting viability over 95%.

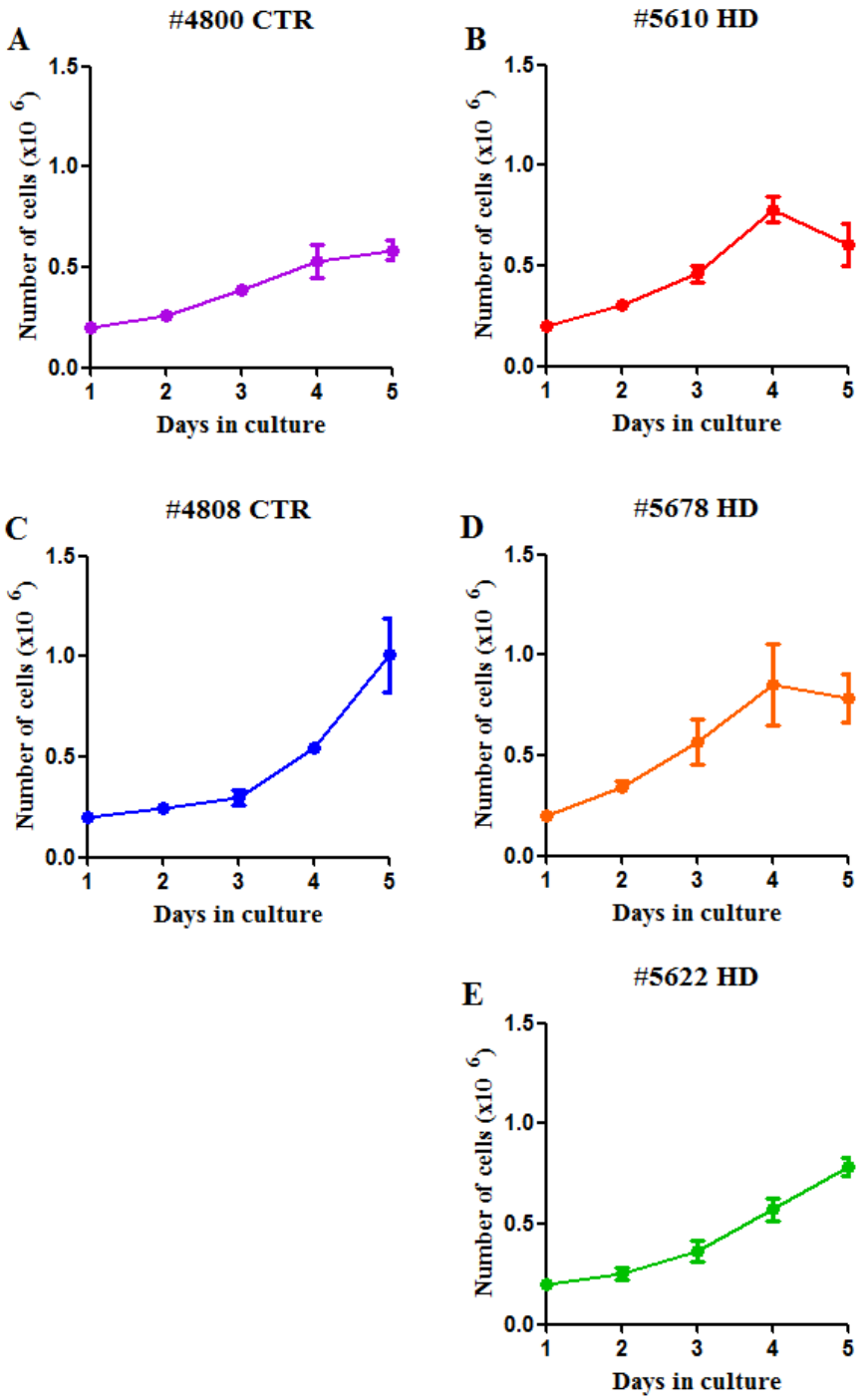


Figure 2.2. Proliferation curves of CTR or HD lymphoblast cell lines. CTR (A and C) or HD lymphoblasts (B, D and E) were seeded at a density of $0.2x10^6$ cells/ml and cultured for 5 days in the incubator chamber. Every day the cells were evaluated by trypan blue assay and counted by using an hemocytometer. Results are presented as mean ± SEM of 3-4 independent experiments performed in duplicates.

2.5. CREATION OF ρ^0 CELL LINES

Rho-zero cell lines from CTR or HD lymphoblasts were produced by culturing lymphoblasts in RPMI-1640 medium supplemented with 100 µg/ml sodium pyruvate and 100 µg/ml uridine (ρ^0 growth medium) [to maintain selection of cells defective in respiration, which can be auxotrophic for these substrates] in the presence EtBr, a cationic and lipophilic agent that, when present in small amounts, inhibits mtDNA replication and transcription leading to mtDNA damage (King & Attardi, 1989, Miller *et al.*, 1996). EtBr exposure results in pyrimidine auxotrophy, since it was previously demonstrated that cells maintained for 4 months in 25 ng/ml EtBr lack ETC function and died in medium not supplemented with pyruvate and uridine (Swerdlow *et al.*, 1997) and also reproduced (Desjardins *et al.*, 1985, King & Attardi, 1989), which can be overcome by supplementing cells with pyruvate and uridine Absence of OXPHOS, due to EtBr exposure, deprives cells of the major NADH oxidation pathway. On the other hand, uridine-dependence is the result of inactive dihydroorotate dehydrogenase, which is dependent on the respiratory chain for recycling of flavin adenine dinucleotide (FAD). However, it was also observed lack of uridine auxotrophy in SH-SY5Y cells treated with 5 µg/ml of EtBr for 64 days, suggesting that functional dihydroorotate dehydrogenase or residual uridine in the dialyzed FBS may be responsible for cell survival in the absence of added uridine (Miller *et al.*, 1996).

Cells were seeded in ρ^0 growth medium plus EtBr at density of $0{,}4x10^6$ cell/ml and cultured for 15 or 30 days, as indicated in figure legends. Cells were replated twice a week with fresh EtBr supplemented ρ^0 growth medium. After 15 or 30 days of drug treatment, cells were cultured in EtBr-free ρ^0 growth medium in order to achieve the presence of remained aerobic competent in cultured cells. Control cells were maintained in culture for the same period of time in PRMI 1640 medium, in the absence of EtBr.

2.6. SUBCELLULAR FRACTIONATION

2.6.1. TOTAL EXTRACTS

Cells were gently ressuspended in order to dissociate the aggregates and washed by centrifugation at 850 rpm for 5 min at 4ºC in ice-cold phosphate-buffered saline (PBS) containing (in mM) 137 NaCl, 2.7 KCl, 1.8 KH_2PO_4, 10 $Na_2HPO_4.2H_2O$, pH 7.4, and further extracted in lysis buffer (150 mM Nacl, 50 nM Tris, 5 mM EGTA, 1%

Triton X-100; 0,5% DOC, 0,1% SDS, pH 7.5) supplemented with 1 mM DTT, 1 mM PMSF and a 1:1000 protease cocktail inhibitor (chymostatin, pepstatin A, leupeptin and antipain). Cellular extracts were centrifuged at 14000 rpm for 10 min (Eppendorf Centrifuge 5417R) to remove cellular debris. The pellet was discarded and the supernatant (total extract) was collected and protein content quantified by Bio-Rad method (BioRad, Hercules, CA, and USA). Extracts were stored at -80ºC until used for western blotting experiments.

2.6.2. MITOCHONDRIAL EXTRACTS

After being collected, cells were washed by centrifugation at 850 rpm for 5 min at 4ºC in ice-cold PBS, ressuspended in sucrose buffer (250 mM, 20 mM HEPES, 10 mM or 100 mM KCl, 1.5 $MgCl_2$, 1 mM EDTA, 1 mM EGTA pH 7.5/KOH), homogenized 20x by using a potter (3431E06, Thomas Scientific) and centrifuged at 2,300 rpm for 5 min. The supernatant (mitochondrial fraction) was collected and assayed for protein content by BioRad method and stored at -80ºC until used for mitochondrial respiratory chain complex activities by using a UV/VIS spectrophotometer (model 2401; Shimadzu Scientific Instruments, Columbia, MD).

2.7. MITOCHONDRIAL RESPIRATORY CHAIN COMPLEXES ACTIVITIES

2.7.1. NADH-UBIQUINONE OXIDOREDUCTASE ASSAY

Cx I activity was determined by a modified version of Ragan et al. (1987), which follows the decrease in NADH absorbance at 340 nm that occurs when ubiquinone (CoQ1) is reduced to ubiquinol. The reaction was started by adding the sample to the reaction mixture (in mM: 20 K_2HPO_4, pH 7.2, 10 $MgCl_2$, 0.15 NADH, 2.5 mg/ml BSA fatty-acid free, 1 KCN) containing 50 μM decylubiquinone, at 30ºC. After 8 min, rotenone (10 μM) was added and the reaction was registered for further 10 min. Cx I activity was expressed in nanomoles per minute per milligram of protein and correspond to the rotenone sensitive rate.

2.7.2. CYTOCROME C OXIDASE ASSAY

Cx IV activity was determined using the method of Wharton and Tzagotoff (1967), which measures the oxidation of reduced citocrome c by citochrome c oxidase at 550 nm. The reduced cytochrome c was prepared by mixing its oxidised form with a

few crystals of ascorbate and then dialysed membrane (Medicell International) for 3 days against 0,01 phosphate buffer, and pH 7.0 at 4 º. The reduced cytochrome c concentration was then determined using 0,1 M potassium ferricyanide. The assay was started by adding the sample to the reaction buffer (10 mM K_2HPO_4, pH 7.0) containing 50 μM reduced cytochrome c and 1 mM ferricyanide, at 30ºC. Cx IV activity was expressed in rate constant (k) per minute per milligram of protein.

2.8. WESTERN BLOTTING ANALYSIS

Total extracts obtained as described in 3.6.1. were denatured with 6 times concentrated denaturing buffer (300 mM Tris-HCl pH 6.8, 12% SDS, 30% glycerol, 600 mM DTT, 0.06% bromophenol blue) at 95 ºC, for 5 min. Equivalent amount of protein were separated on a 7,5-12% SDS-PAGE gel electrophoresis and electroblotted onto polyvinylidene difuoride (PVDF) membranes (Milipore, Billerica, MA, USA). The membranes were blocked for 1 h in Tris-buffered saline (TBS) solution containing 0,1% Tween (TBS-Tween) with 5% fat-free milk, followed by an overnight incubation with primary antibodies against Cx I 20 kDa subunit (1:500), Cx I 30 kDa subunit (1:250), Cx II 70 kDa subunit (1:1000), Cx IV subunit I (COX-1) (1:300) or HSP60 (1:250), at 4ºC with gentle agitation. Membranes were then washed 3x for 15 min with TBS-Tween, and incubated with anti-mouse secondary antibody (1:20000) for 2h, at room temperature with gentle agitation. Immunoreactive bands were visualized by alkaline phosphatase activity that catalyses the conversion of ECF substrate to a highly fluorescent product reagent that strongly fluoresces at 540–560 nm when the blots are illuminated with UV light (maximum excitation at 430 nm), by using a BioRad Versa Doc 3000 Imaging System.

2.9. DATA ANALYSIS AND STATISTICS

Results are expressed as mean ± SEM of the indicated number of independent experiments. Comparisons between multiple groups were performed with a two-way analysis of variance (ANOVA), followed by Dunnett´s post-test for comparison between experimental groups. Significance was accepted at $p<0.05$. Comparison between two groups was achieved with Student´s *t* test.

-CHAPTER 3. RESULTS-

The goal of the present study was to create and characterize ρ^0 cells from CTR or HD lymphoblastoid cell lines. In the first part of this work, we analyzed the activity of Cx I and Cx IV of the MRC and also the expression of both mitochondrial- and nuclear-encoded subunits in CTR or HD lymphoblast cells. This procedure was also done in CTR or HD lymphoblasts after EtBr treatment, in order to confirm the ρ^0 *status*. In addition, we tested the expression of Hsp60, a nDNA-encoded mitochondrial protein in cells exposed to EtBr. Finally ρ^0 *status* of our culture model was evaluated 60 days after EtBr withdrawal but in the presence of uridine and pyruvate.

In preliminary studies we used EtBr concentrations from 25 up to 5000 ng/ml (25, 100, 500, 1000 and 5000 ng/ml). We observed a decrease in cell viability in both CTR and HD lymphoblast exposed to concentrations of EtBr above 25 ng/ml, suggesting that these concentrations are toxic and lethal for lymphoblasts (data not shown). Both 25 and 50 ng/ml EtBr were then tested, and despite the fact that 25 ng/ml EtBr treatment lead to a slight decrease in the proliferation rate of both CTR and HD lymphoblast cell lines, this decrease was more pronounced in 50 ng/ml EtBr-treated cells, but without significantly affecting cell viability (data not shown).

3.1. ELECTRON RESPIRATORY CHAIN FUNCTION IN CTR AND HD LYMPHOBLASTS

The OXPHOS system of MRC has a dual genetic origin, since the Cx I, Cx III and Cx IV include both nDNA- and mtDNA-encoded subunits, whereas Cx II is made entirely of nDNA-encoded subunits (Walker, 1995).Results depicted in Figure 3.1 show that the basal expression levels of both mtDNA-encoded Cx I 20 kDa (A), and Cx IV 57 kDa (C) subunits and also the nDNA-encoded Cx I 30 kDa (B) and Cx II 70 kDa (D) subunits, are not different significantly between CTR and HD lymphoblasts. These results suggest that the Htt mutation present in HD lymphoblasts, *per se,* does not affect the expression levels of these subunits.

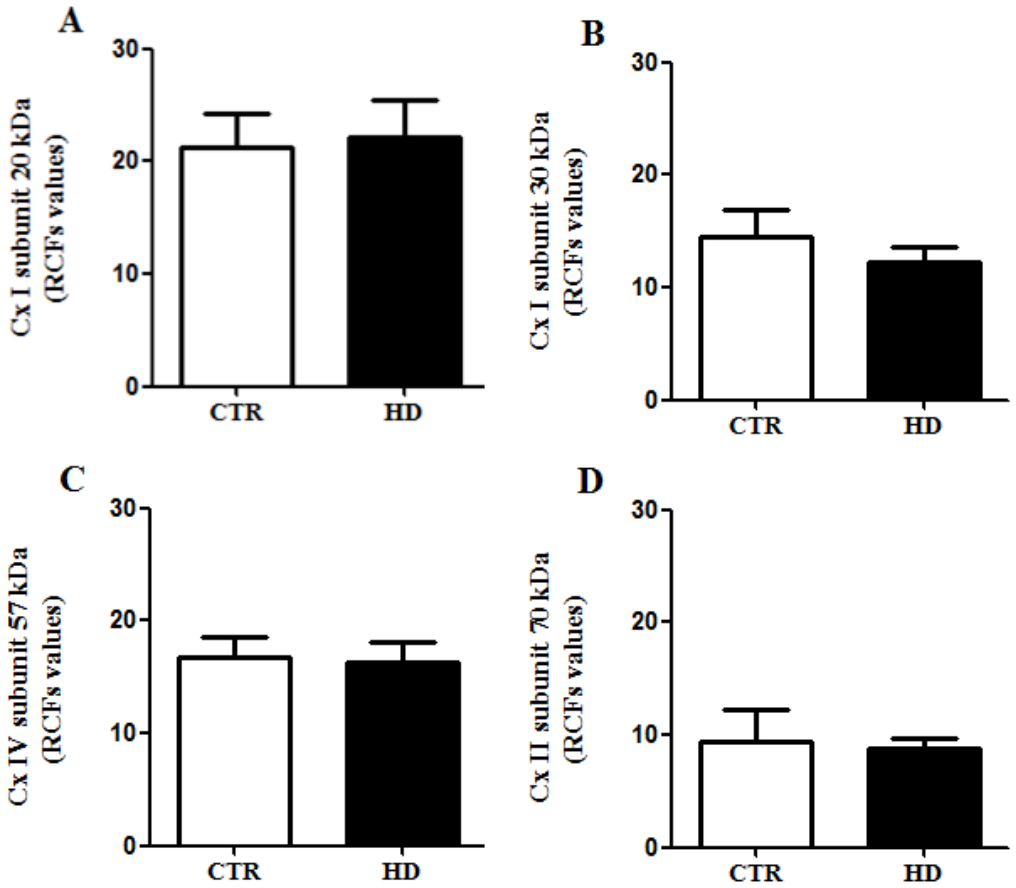


Figure 3.1. Expression levels of mtDNA- and nDNA-encoded MRC subunits in CTR and HD lymphoblasts. Cell lines were cultured and total cellular extracts performed as described in Material and Methods. (A) Cx I mitochondrial 20 kDa subunit; (B) Cx I nuclear 30 kDa subunit; (C) Cx IV mitochondrial 57 kDa subunit and (D) Cx II 70 kDa subunit expression values. Data are expressed as the mean±SEM from 3-12 independent experiments.

We then analyzed the MRC complex activities, namely the NADH ubiquinone oxidoreductase (Cx I) and cytochrome c oxidase (Cx IV), both of which contain subunits encoded by mtDNA and nDNA (Fig. 2). We observed that the activity of Cx I is slightly increased, in a non significant manner, in HD lymphoblasts (Fig. 3.2 A) but no differences were observed in Cx IV activity between CTR and HD lymphoblasts (Fig. 3.2 B).

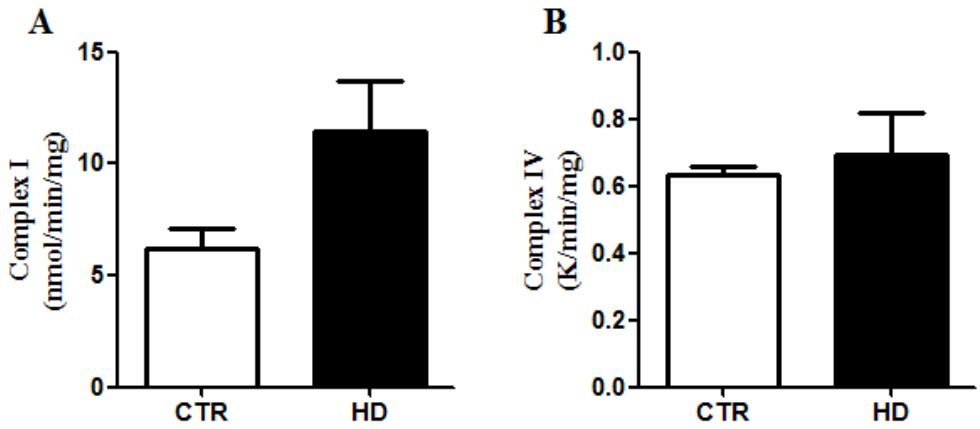


Figure 3.2. MRC Cx I and Cx IV activities in lymphoblast cell lines. The activities of Cx I (A) and Cx IV (B) were measured in basal conditions in CTR or HD lymphoblasts, cultured and extracted as described in Material and Methods. Data are expressed as the mean±SEM from 3-8 independent experiments.

3.1.1. EFFECT OF ETHIDIUM BROMIDE IN THE EXPRESSION OF MITOCHONDRIAL CHAIN SUBUNITS ENCODED BY MTDNA

In order to produce the ρ^0 *status* of both CTR and HD lymphoblasts, cells were exposed to low concentrations of EtBr which was extensively used to reduce the mtDNA copy number in proliferating cells (Desjardins *et al.*, 1986) by inhibiting mitochondrial transcription and replication systems (Seidel-Rogol & Shadel, 2002), without substantially affecting nDNA. Our results demonstrate that exposure to 25 and 50 ng/ml EtBr during 15 (Fig. 3.3 A and B) or 30 days (Fig. 3.3 C and D) significantly decreased the expression of mtDNA-encoded Cx I 20 kDa subunit in CTR (Fig. 3.3 A and C) and HD lymphoblasts (Fig. 3.3 B and D).

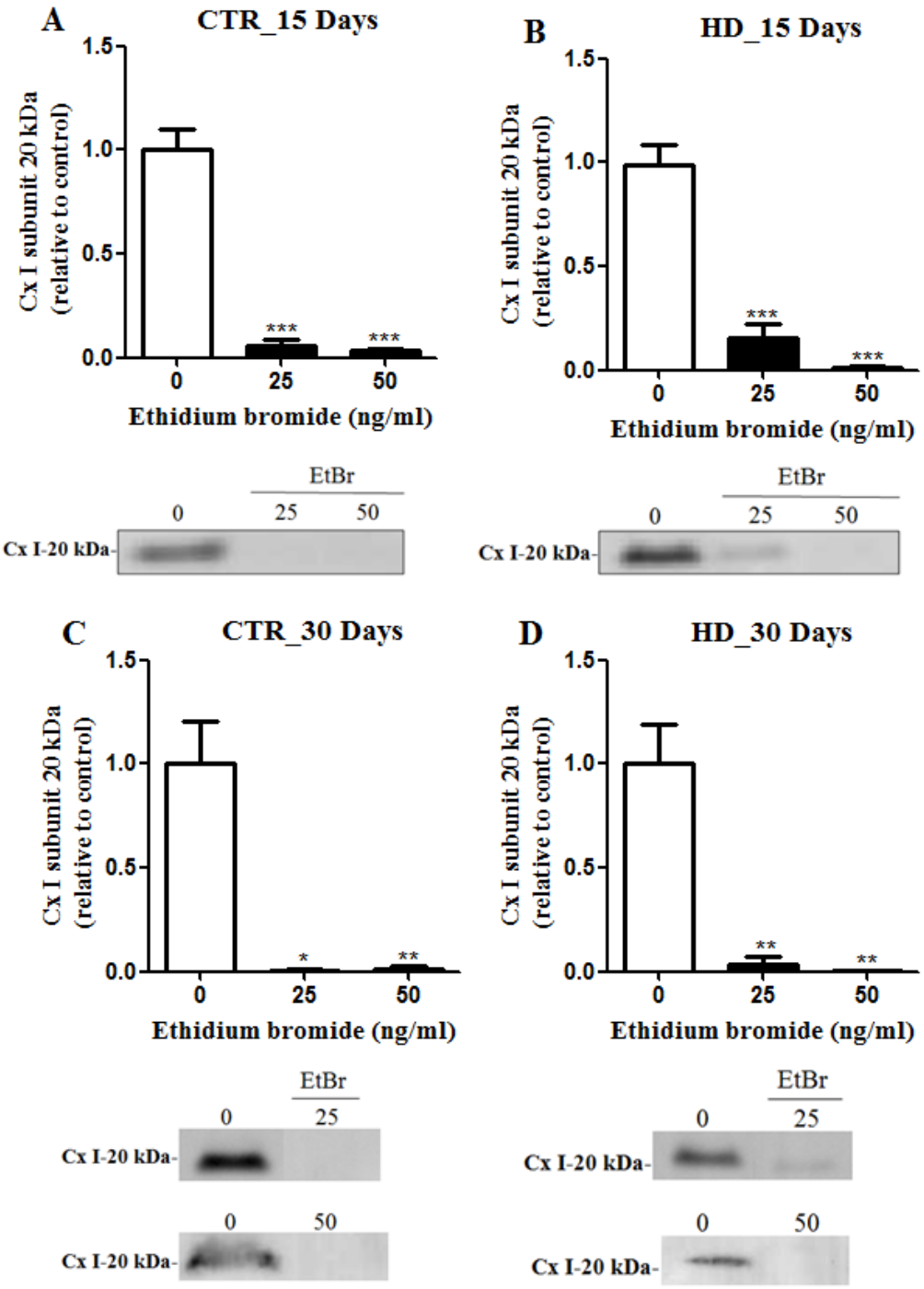


Figure 3.3 Effect of EtBr on the expression levels of mtDNA-encoded Cx I 20 kDa subunit. CTR or HD lymphoblast were cultured in the absence or in the presence of 25 and 50 ng/ml EtBr for 15 days (A and B) or 30 days (C and D) and total extracts performed as described in Materials and Methods. Data are expressed as the mean±SEM from 2-8 independent experiments normalized for control conditions, in the absence of EtBr. *p<0.05; **p<0.01; ***p<0.001 with respect to controls, in the absence of EtBr.

Since we observed a significant decrease in mtDNA-encoded Cx I 20 kDa subunit in both CTR and HD lymphoblasts exposed to 25 or 50 ng/ml EtBr during 15 days, the expression levels of mtDNA- encoded Cx IV 57 kDa subunit were also evaluated, in the same conditions (Fig. 3.4). Our results demonstrate a significant, although not complete, decrease in the expression of Cx IV 57 kDa subunit in CTR cells exposed to 25 and 50 ng/ml EtBr (Fig. 3.4 A). We did not observe any changes in the Cx IV 57 kDa subunit expression when the HD cells were exposed to 25 ng/ml EtBr; however, a slight but not significant decrease in the expression levels of this subunit was observed in HD lymphoblasts exposed to 50ng/ml EtBr (Fig. 3.4 B), suggesting that EtBr was not able to reduce Cx IV 57 kDa subunit expression levels, as observed for CxI-associated subunit presented in Figure 3.3. Unfortunately, we have some difficulties in using the antibody against Cx IV 57 kDa subunit in cells treated for 30 days with EtBr.

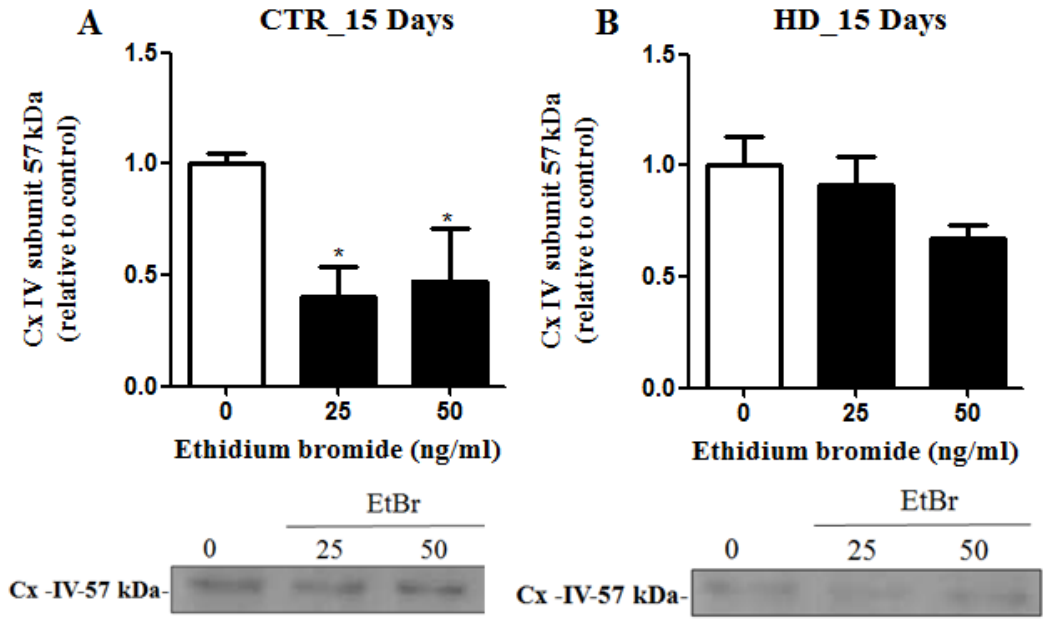


Figure 3.4 Effect of EtBr on the expression levels of mtDNA-encoded Cx IV 57 kDa subunit. CTR (A) or HD (B) lymphoblasts were cultured in the absence or in the presence of 25 and 50 ng/ml EtBr for 15 days and total extracts performed as described in Materials and Methods. Data are expressed as the mean±SEM from 3-6 independent experiments and results were normalized for control conditions.*$p<0.05$ in comparison to the control, in the absence of EtBr.

3.1.2. ETHIDIUM BROMIDE TREATMENT DOES NOT AFFECT EXPRESSION OF MITOCHONDRIAL RESPIRATORY CHAIN SUBUNITS ENCODED BY NDNA

Since low concentrations of EtBr inhibit mtDNA synthesis without affecting nDNA synthesis, we analyzed the levels of expression of MRC nDNA-encoded subunits, namely Cx I 30 kDa (Fig 3.5) and Cx II 70 kDa subunit (Fig. 3.6). Our results demonstrate that 25 or 50 ng/ml EtBr did not affect expression levels of nDNA-encoded Cx I 30 kDa subunit in both CTR (Fig. 3.5 A and C) or HD lymphoblasts (Fig. 3.5 B and D) when exposed for 15 or 30 days.

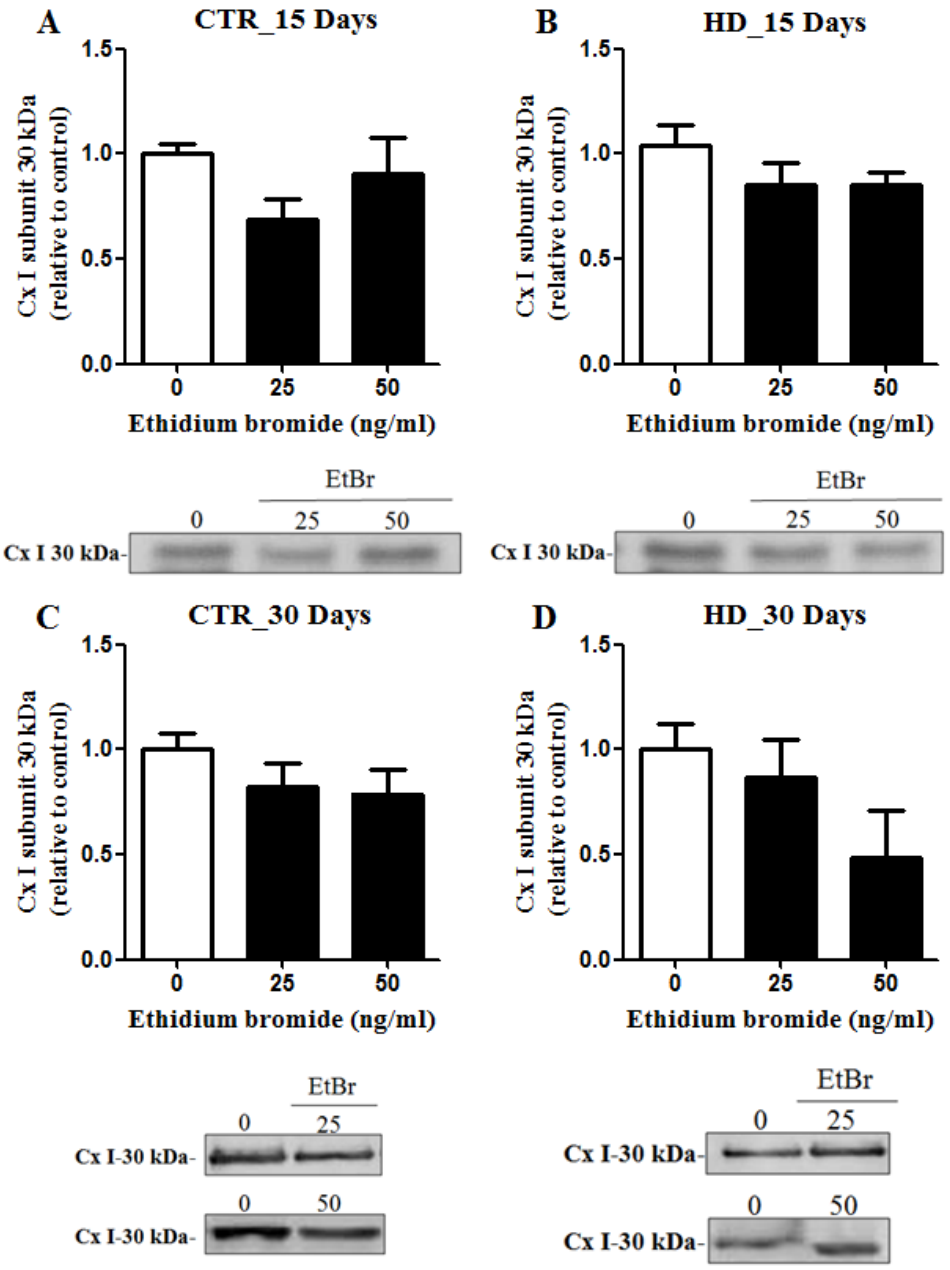


Figure 3.5 Effect of EtBr on the expression levels of nDNA-encoded Cx I 30kDa subunit. Cells were cultured in the absence or in the presence of 25 and 50 ng/ml EtBr for 15 days (A and B) or 30 days (C and D) and total extracts performed as described in Materials and Methods. Data are expressed as the mean±SEM from 2-7 independent experiments and the results were normalized for control conditions.

Results depicted in Figure 3.6 demonstrate that EtBr in the concentrations tested did not affect nDNA-encoded 70 kDa subunit of Cx II in CTR (Fig.3.6 A and C) or in HD (Fig. 3.6 B and D) cells, subjected to EtBr for 15 or 30 days. Altogether these results suggest that, in our experimental conditions, EtBr decreased the expression levels of mtDNA-encoded subunits without affecting nDNA-encoded subunits of MRC Cx I, Cx II and Cx IV.

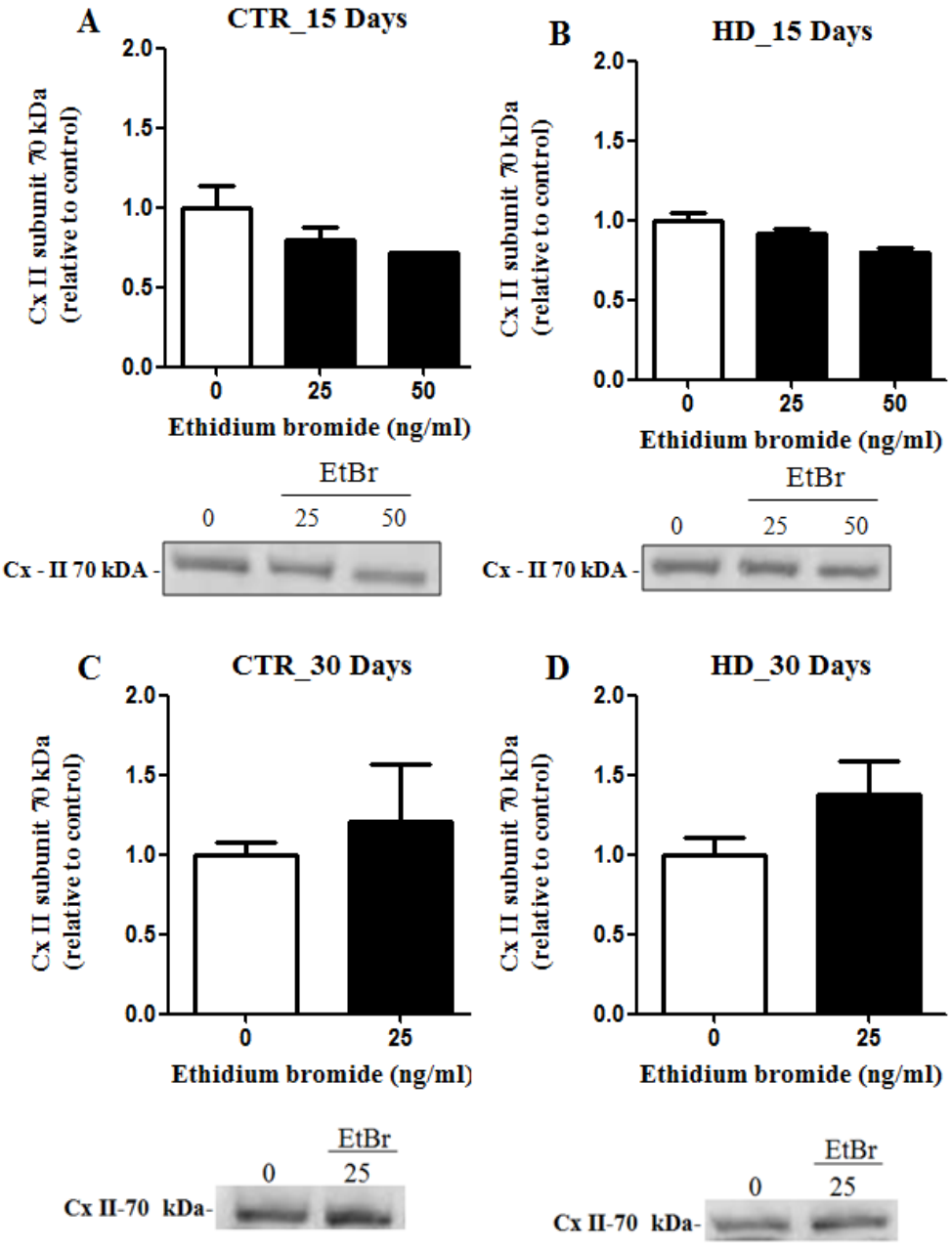


Figure 3.6. Effect of EtBron the expression levels of nDNA-encoded Cx II 70kDa subunit. Cells were cultured in the absence or in the presence of 25 and 50 ng/ml EtBr for 15 days (A and B) or 30 days **(**C and D) and total extracts performed as described in Materials and Methods. Data are expressed as the mean±SEM from 1-6 different experiments and results were normalized for control conditions.

3.2. ETHIDIUM BROMIDE TREATMENT DOES NOT INFLUENCE THE LEVELS OF NDNA-ENCODED MITOCHONDRIAL PROTEIN HSP60

In order to evaluate the putative effect of EtBr on the expression levels of another mitochondrial protein codified by nuclear genome, not associated with the MRC, the expression levels of Hsp60 protein, a chaperone involved in the folding of proteins imported into the mitochondria, was also analyzed. Under basal conditions, no differences were observed between CTR and HD lymphoblasts (data not shown). As shown in the Figure 3.7, no changes was observed in both CTR (Fig. 3.7 A and C) and HD (Fig. 3.7 B and D) lymphoblast cells treated with EtBr 25 and 50 ng/ml for 15 or 30 days. These results again point out for the absence of EtBr in affecting nDNA-encoded proteins under the experimental conditions used in this work.

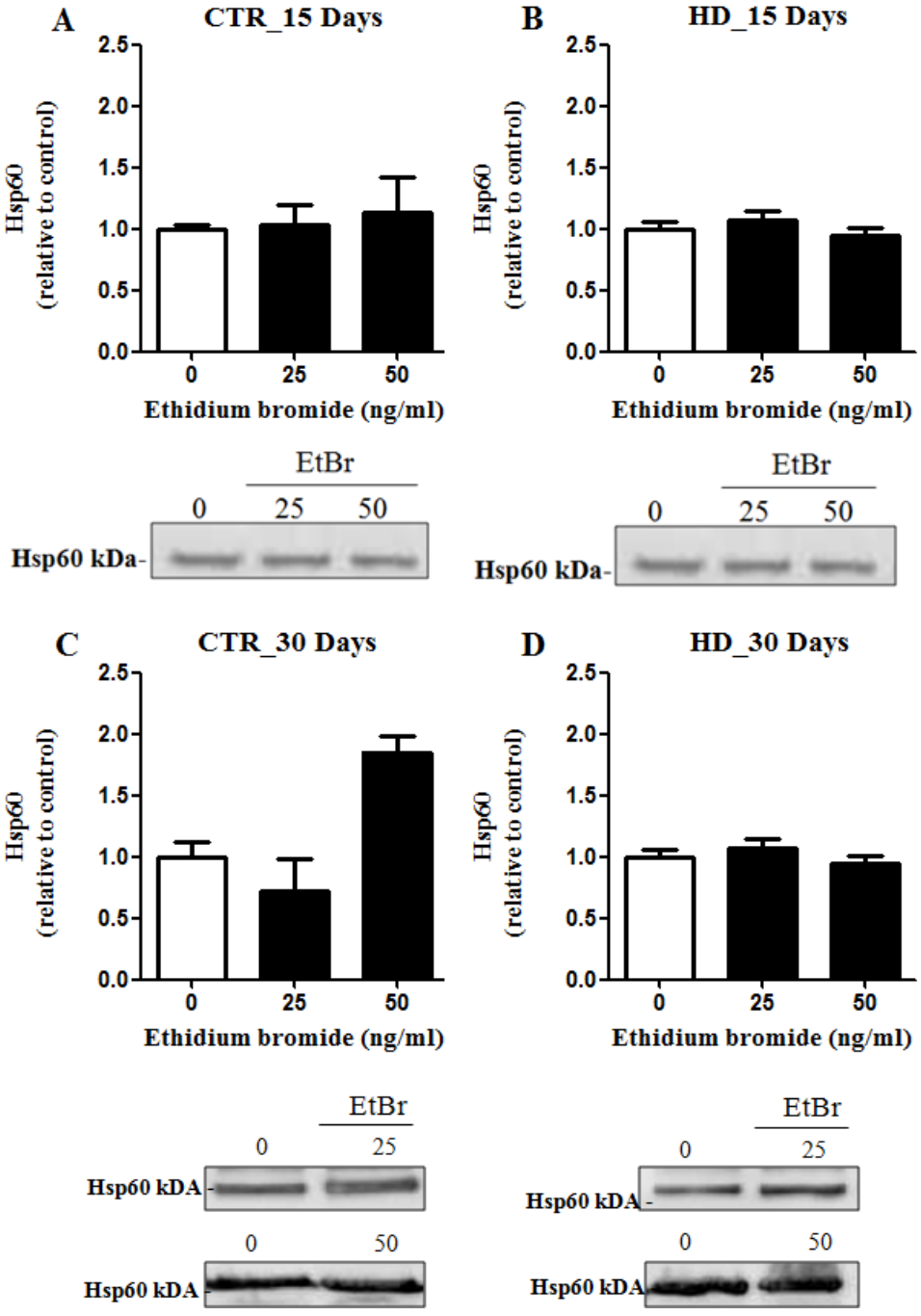


Figure 3.7. Effect of EtBr on the expression levels of nDNA-encoded Hsp60. Cells were cultured in the absence or in the presence of 25 and 50 EtBr for 15 days (A and B) or 30 days (C and D) and total extracts performed as described in Materials and Methods. Data are expressed as the mean±SEM from 2 - 12 independent experiments and results were normalized for control conditions (in the absence of EtBr).

Our results demonstrate that EtBr significantly decreased the expression levels of mtDNA-encoded 20 kDa subunit without affecting the nDNA-encoded 30 kDa subunit associated with Cx I. However, the decrease in mtDNA-encoded subunit 57 kDa associated with Cx IV was shown not to be so evident. Thus, the activities of

NADH ubiquinone oxidoreductase (Cx I) and cytochrome c oxidase (Cx IV) in CTR and HD lymphoblasts subjected to EtBr exposure were evaluated. Data depicted in Figure 3.8 demonstrate that exposure to 25 ng/ml EtBr for 15 days significantly decreased Cx I activity in both CTR and HD lymphoblasts (Fig. 3.8 A and B). The same response was observed in HD lymphoblasts treated with EtBr for 30 days (Fig. 3.8 D). Unfortunately, we did not observe a decrease in Cx I activity in CTR lymphoblasts treated for 30 days, which can be due to the reduced number of experiments performed (n=1) (Fig. 3.8 C).

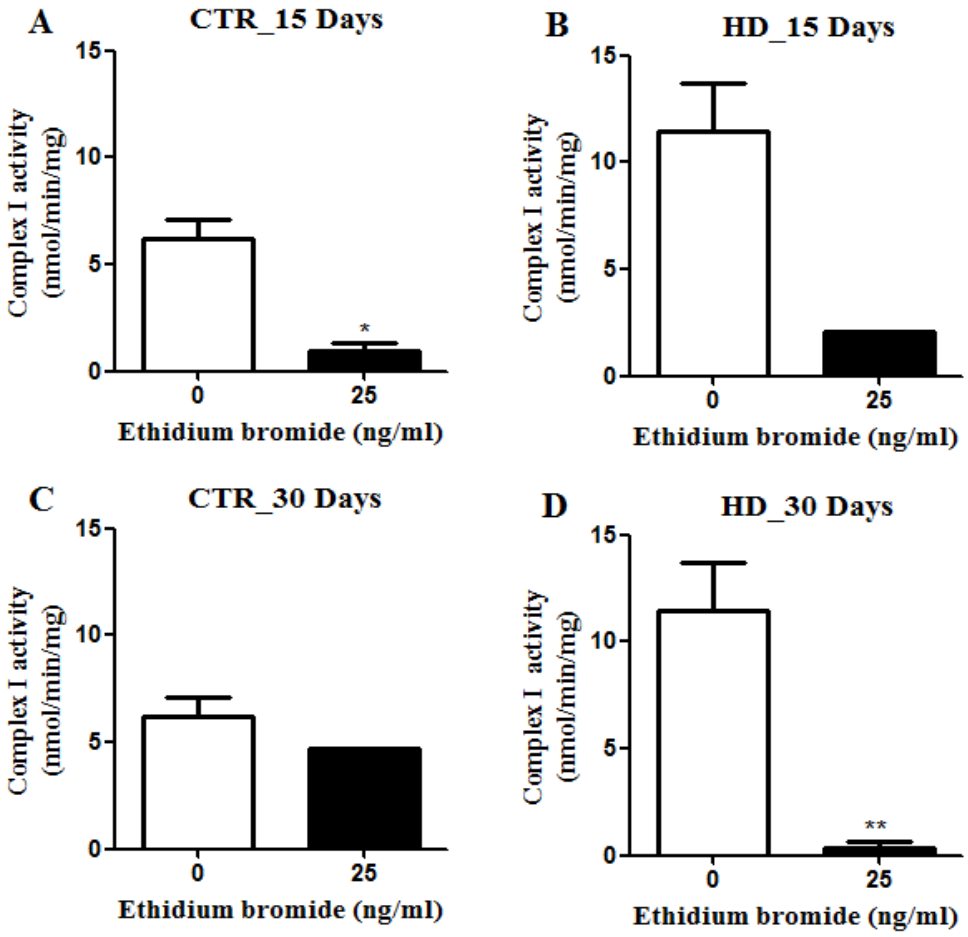


Figure 3.8. Effect of ethidium bromide on the MRC Cx I activity. Cells were cultured in the absence or in the presence of 25 ng /ml EtBr for 15 days (A and B) or 30 days (C and D). Mitochondrial extracts were performed as described in Materials and Methods. Data are expressed as the mean±SEM from 1-4 independent experiments. *p<0.05 or **p<0.01 in comparison to the control, in the absence of EtBr.

We next examined the activity of Cx IV in both CTR and HD lymphoblasts exposed to EtBr in the same conditions as described previously. Our results demonstrate that EtBr significantly decreased Cx IV activity in both cell lines (Fig. 3.9) being this affected more pronounced when the cells were treated during 30 days to EtBr (Fig 3.9 C and D).

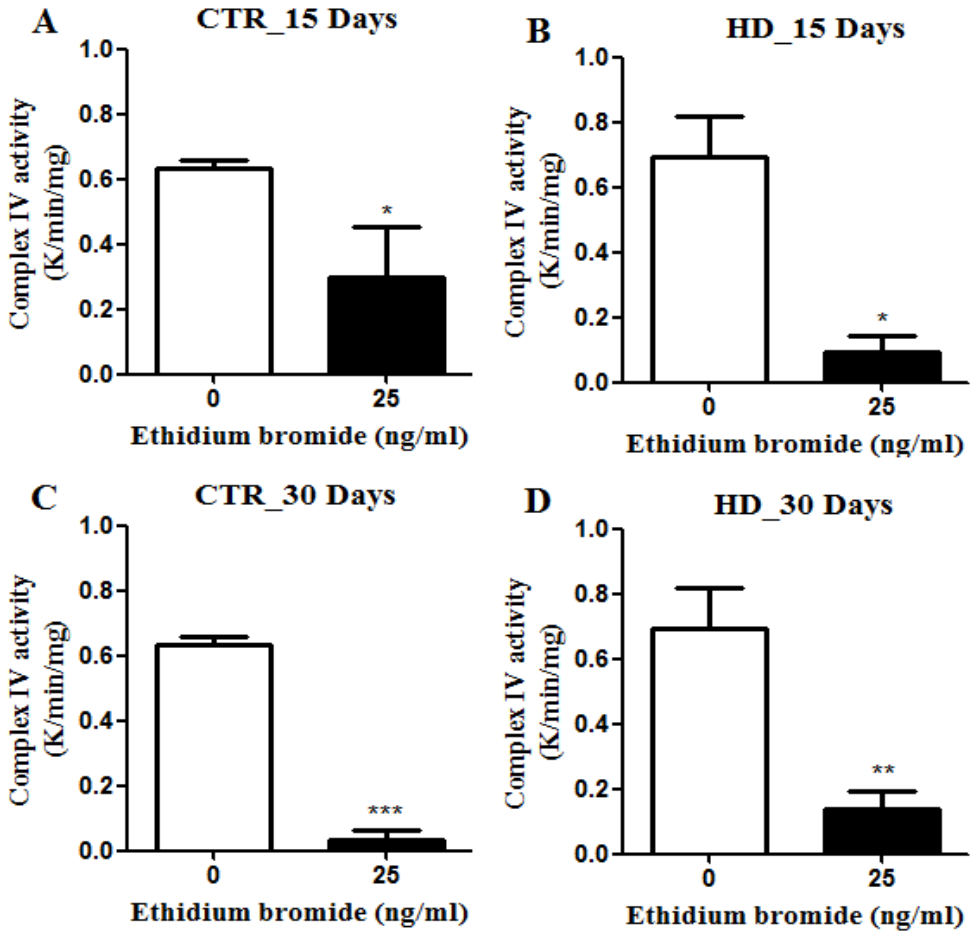


Figure 3.9. Effect of ethidium bromide on the MCR Cx IV activity. Cell lines were cultured in the absence or in the presence of 25ng /ml EtBr for 15 days (A and B) or 30 days (C and D). Mitochondrial extracts performed as described in Materials and Method. Data are expressed as the mean±SEM from 3-8 independent experiments. *p<0.05; **p<0.01; ***p<0.001 with respect to the control, in the absence of EtBr.

3.3. CELL REPOPULATION – RECOVERED EXPRESSION OF MITOCHONDRIAL ENCODED SUBUNITS OF COMPLEX I AND IV AFTER ETHIDIUM BROMIDE WITHDRAWAL

Strong selection of 143B.206 cells defective in respiration has been described to occur after 60 days in the presence of uridine and pyruvate, and in the absence of EtBr (King & Attardi, 1989). Moreover, a ρ^0 status is needed for at least 2 months in order to perform further experiments. Thus, using this period of time, we evaluated the expression levels of mtDNA-encoded subunits, as shown in Figure 3.10, which includes a replot of Figures 3.3 and 3.4**.** Our results show that the observed decrease in mtDNA-encoded subunits of both Cx I (20 kDa) and Cx IV (57 kDa) in response to EtBr exposure was reverted by culturing both CTR and HD lymphoblasts, for 60 days, in uridine and pyruvate-supplemented culture medium without EtBr. In fact, as shown in Figure 3.10, the expression levels of Cx I mtDNA-encoded 20 kDa subunit recovered by about 40% when CTR lymphoblasts were cultured in the absence of EtBr, compared with the mean value achieved by exposure to 25 ng/ml EtBr for 30 days (Fig. 3.10 A). Surprisingly, the expression levels of Cx I mtDNA-encoded 20 kDa subunit in HD lymphoblasts in cells incubated for further 60 days 0 EtBr increased by about 40% over the basal (Fig. 3.10 B). In the same manner, the expression values of Cx IV mtDNA-encoded 57 kDa subunit of CTR lymphoblasts cultured for additional 60 days in EtBr-free medium recovered to control levels (Fig. 3.10 C). Despite the observation of no significant effect of EtBr in reducing the expression levels of Cx IV mtDNA-encoded 57 kDa subunit in HD lymphoblasts (Fig. 3.10 D; see also Fig. 3.4 B), a slight, although not significant increase in the expression levels of Cx IV mtDNA-encoded 57 kDa subunit was observed, as compared to basal levels (Fig.3.10 D).

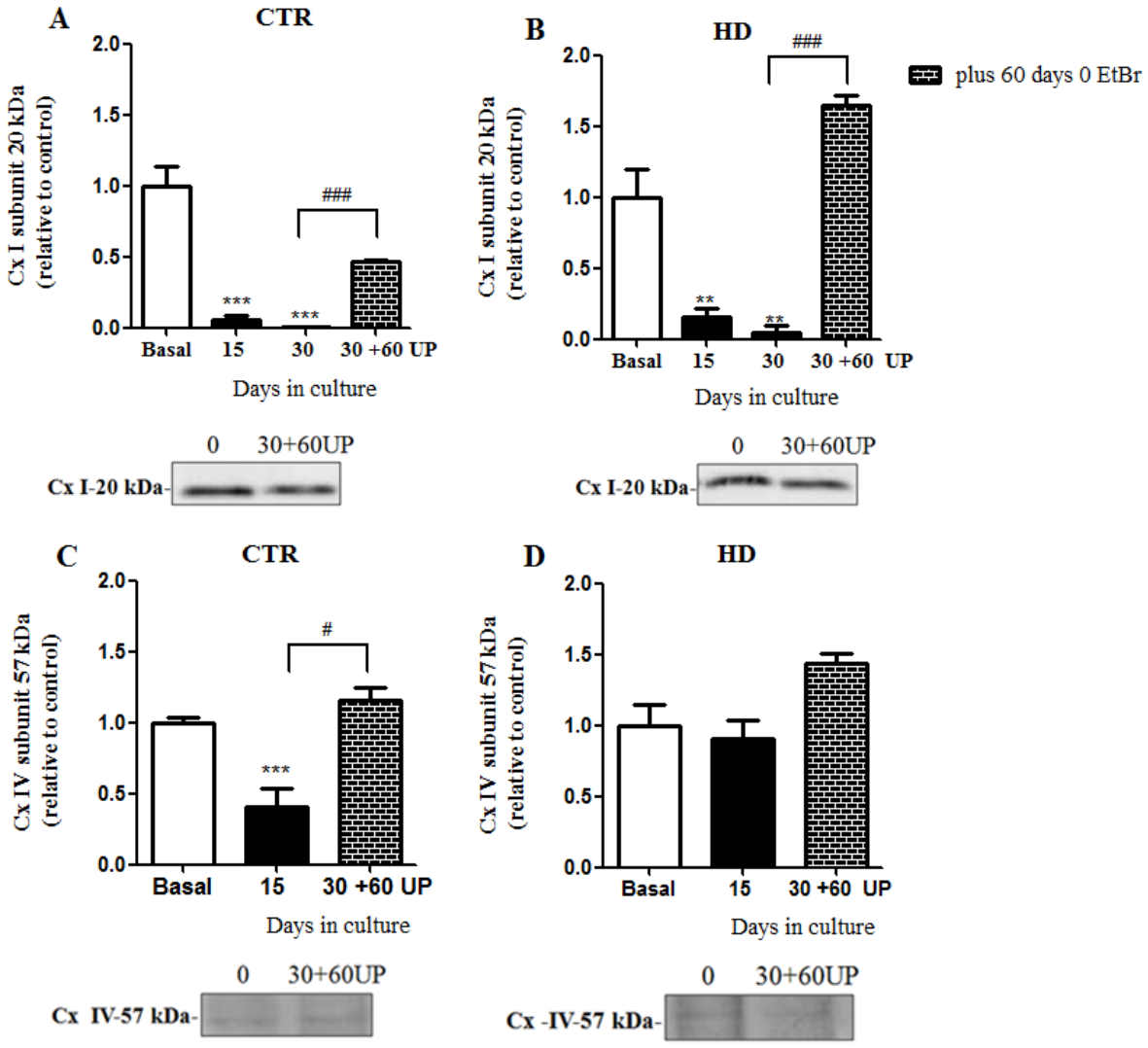


Figure 3.10. Effect of ethidium bromide withdrawal on the expression levels of Cx I and Cx IV mtDNA-encoded subunits. Cells were cultured for 15 or 30 days in the ρ^0 growth medium in the presence of 25 ng/ml EtBr, and then cultured for more 60 days in uridine/pyruvate-supplemented culture medium without added EtBr (0EtBr). Total extracts were performed as described in Materials and Methods. Data are expressed as the mean±SEM from at least 2-10 different experiments and normalized for control conditions. **p<0.01;*** p<0.001 with respect to controls (or basal conditions, in the absence of EtBr) and # p<0.05; ### p<0.001 in comparison to the same condition following EtBr exposure.

-CHAPTER 4. DISCUSSION-

Mutant huntingtin is known to be widely expressed not only in the brain but also in peripheral tissues, suggesting that an adverse effect of mHtt is not limited to neurons (Sassone *et al.*, 2009). Indeed, human HD lymphoblasts have provided clear-cut data on mitochondrial disruption. Lymphoblasts have been used in many studies as a cellular model of HD, to understand the mechanisms related to this disease, namely regarding apoptotic cell death (Sawa *et al.*, 1999), impaired mitochondrial Ca^{2+} buffering (Panov *et al.*, 2002), mitochondrial ultrastructural changes, altered mitochondrial membrane potential (Squitieri *et al.*, 2006) and also genetic instability (Cannella *et al.*, 2009, Squitieri *et al.*, 2006). Results of our group also demonstrated that lymphocytes obtained from peripheral blood of HD patients may reflect changes observed in HD brain (Almeida *et al.*, 2008, Panov *et al.*, 2002). Moreover, mHtt fragments can accumulate on the mitochondrial membrane, causing mitochondrial dysfunction. In fact, mitochondria isolated from lymphoblasts of HD patients have a lower membrane potential and despolarize at lower Ca^{2+} loads than mitochondria from CTR lymphoblasts (Panov *et al.*, 2002).

The goal of the present study was to create and characterize a new human cell model for studying mitochondrial dysfunction in HD, namely CTR *versus* HD ρ^0 lymphoblasts cells. Accordingly to King and Attardi (1989), chronic exposure to low concentrations of EtBr depletes cells from mtDNA without affecting nDNA. It was previously described that during exposure to EtBr, circular mtDNA breaks down (Miller *et al.*, 1996) leading to subsequent selective inhibition of mtDNA-encoded protein synthesis, a reduction in the cell growth with a concomitant progressive dilution of the number of mtDNA per cell (Wiseman & Attardi, 1978). Creation of ρ^0-derived mammalian cells using EtBr has been described for neuroblastoma cells SY-SY5 (Miller *et al.*, 1996, Swerdlow *et al.*, 1997), NT-2 cells (Swerdlow *et al.*, 1997), chicken fibroblasts (Desjardin et al., 1985) and lung carcinoma or osteosarcoma cells (King & Attardi, 1989). However, until now, no data have been reported in producing ρ^0 cells derived from human lymphoblasts.

In the present work both 25 and 50 ng/ml EtBr decreased lymphoblasts MRC associated function when the cells were exposed to EtBr for 15 or 30 days. In fact, treatment of SH-SY5Y neuroblastoma cells with 5 µg/ml (Miller *et al.*, 1996) and NT2 teratocarcionama cell lines with 25 ng/ml EtBr was used previously to produce a ρ^0 *status* (Swerdlow *et al.*, 1997), while treatment with 50 ng/ml was used to produce

fibroblast, osteosarcoma and rhabdomyosarcoma ρ^0 cell lines (King & Attadi, 1996, Vergani *et al.*, 2000). These results suggest that sensitivity to EtBr treatment may be cell type specific and therefore must be adjusted for each cell type.

As described in Results section, exposure to 25 and 50 ng/ml EtBr caused a slight decrease in the proliferation rates of CTR and HD lymphoblasts, although not affecting cell viability. These observations are in accordance with several studies in ρ^0 cells which exhibit a decrease in proliferation rate compared to wild-type cells (in the absence of any treatment), namely in 143B TK^- cells (King & Attadi, 1996), HeLa cells (Piechota *et al.*, 2006), human leukemia T-cell line (MOLT-4) (Armand *et al.*, 2004) and in the newly established ρ^0 cell line 143B.TK^-K7 (Kukat *et al.*, 2008). The observed decrease in proliferation may be due to an insufficient capacity of the glycolytic pathway to generate ATP in order to compensate the decrease in OXPHOS system (Piechota *et al.*, 2006).

Our results demonstrate that the basal levels of both mtDNA-encoded Cx I 20 kDa and Cx IV 57 kDa subunits and also nDNA-encoded Cx I 30 kDa and Cx II 70 kDa subunits, are not different between CTR and HD lymphoblasts, suggesting that mHtt *per se* is not exerting an effect in the levels of expression of these subunits in HD lymphoblasts. The ρ^0 *status* of both CTR and HD lymphoblast cells produced in this work is demonstrated by the decrease in the expression levels of mtDNA-encoded proteins of ETC and the decrease in Cx I and Cx IV activities. Our results demonstrate that mtDNA-encoded, but not nDNA-encoded subunits, are not expressed in cells treated with EtBr and that CTR or HD lymphoblasts cell lines present a similar pattern of decreased expression in response to EtBr treatment. In fact, both cell lines treated with 25 and 50 ng/ml of EtBr during 15 and 30 days evidenced a significant decrease in the expression of mtDNA-encoded Cx I 20 kDa subunit, but not in the nDNA-encoded Cx I 30 kDa subunit. We also observed that EtBr treatment during 15 days induced a tendency for a decrease in Cx IV 57 kDa subunit expression levels in HD lymphoblasts, being this decrease more evident in CTR lymphoblasts. A report from Marusich et al. (1997) demonstrated that mtDNA-encoded subunits of Cx IV were not expressed in ρ^0 cells. Unfortunately, we were not able to obtain results in mtDNA-encoded Cx IV 57 kDa after incubation with EtBr for 30 days. Furthermore, cells treated with EtBr demonstrated unchanged expression of Cx II 70 kDa subunit for both concentrations of EtBr tested (25 and 50 ng/ml). These observations are in accordance with a previous

study performed by Marusich et al. (1997) in which fibroblasts treated with EtBr maintained the expression of nDNA-encoded mitochondrial proteins such as Cx II-associated 70 kDa and 30 kDa subunits. The expression levels of Hsp60, a mitochondrial protein encoded exclusively by nDNA, was shown not to be altered after EtBr treatment either, again suggesting that the concentrations of EtBr used in this work do not significantly affect the expression of nDNA-encoded proteins. Interestingly, exposure to 25 ng/ml EtBr for 30 days slightly (although not significantly) increased the expression levels of Cx II 70 kDa subunit. Although this result needs to be confirmed, namely by also determining the activity of Cx II, increased protein levels of Cx II subunit may reflect a compensatory mechanism by which there is an increased expression of mitochondrial nDNA-encoded proteins. Enhanced mitochondrial biogenesis (e.g. increased expression of NRF-1 and TFAM factors) was previously observed in ρ^0 HeLa cells treated with EtBr for at least 70 passages, but not after a short term inhibition of mitochondrial genome expression. (Miranda *et al.*, 1999). This observation indicates that a relatively long time is required to induce nuclear genome response to decreased expression of the mitochondrial genome (Piechota *et al.*, 2006) .The activity of MRC complexes is another parameter used to evaluate the ρ^0 phenotype establishment since the functional ρ^0 *status* may be indicated by the loss of MRC complexes activities (except Cx II, which is encoded solely by nDNA). Our results demonstrate that Cx I activity is slightly but non-significantly increased in untreated HD lymphoblasts, when compared to CTR lymphoblasts. This observation is not in accordance with other reports showing no differences in Cx I activity in caudate nucleus (Gu *et al.*, 1996) or platelets (Gu *et al.*, 1996, Powers *et al.*, 2007) of HD patients. In contrast, a reduction in the activity of Cx I was reported in HD platelets (Parker *et al.*, 1990) and HD muscle (Arenas *et al.*, 1998). Our results demonstrate that EtBr-treated cells for 15 or 30 days show a significant decrease in Cx I activity in both HD and CTR lymphoblasts which is in accordance with the observed decrease in the expression of mtDNA-encoded subunits**.** A previous study performed in SH-SY5Y-derived ρ^0 cells also evidenced no detectable Cx I activity in cells exposed to EtBr (Miller *et al.*, 1996). Moreover, we observed that Cx IV activity was similar in CTR and HD lymphoblasts under basal conditions, and 25 ng/ml EtBr treatment significantly decreased Cx IV activity in both CTR and HD lymphoblasts. Our results are in accordance with those obtained in HeLa cells treated with the EtBr for 6 days, in which Cx IV activity was

decreased about 95% in comparison with the CTR cells (Piechota *et al.*, 2006), and in MOLT-4 ρ^0 cells, in which a loss of Cx IV activity was observed in cells exposed during 24 days to EtBr (Armand *et al.*, 2004). Moreover, a study performed in ρ^0 SH-SY5Y cells, demonstrated no detectable Cx IV activity after exposure to EtBr during 64 days (Miller *et al.*, 1996).

In addition, our results demonstrated that EtBr-treated CTR or HD lymphoblasts cultured in the absence of EtBr for 60 days, recovered the expression of mitochondrial-encoded subunits of both Cx I and Cx IV, suggesting that mitochondrial repopulation occurred after EtBr withdrawal. One possible explanation for the recovering of mtDNA-encoded protein expression may be due to the fact that mtDNA defects are often heteroplasmic and cells treated with EtBr may contain variable mixtures of defective and wild-type mtDNA or, alternatively, all cells within a population may not reach a ρ^0 phenotype during EtBr treatment, since a few aerobically competent cells may survive in the cell culture (Miller *et al.*, 1996). The presence of aerobically competent cells in a cellular population could compete with ρ^0 genotype cells, being present in higher amounts relative to ρ^0 cells. This may explain the increased expression of mtDNA-encoded subunits observed in this work after EtBr withdrawal. Conversely, by using cell lines containing the osteosarcoma 143B(TK-) nuclear background and various mtDNAs, Diaz et al. (2002) demonstrated that mtDNA with large deletions, but not pathogenic point mutations, repopulates organelles significantly faster than wild-type genomes in the same cell.

In conclusion, this study describes the creation of a new model to study mitochondrial dysfunction in HD, as well as biochemical and cellular mechanisms in cells with depleted mtDNA. Although according to King and Attardi (1996) it is very difficult to obtain ρ^0 derivates of all human cell lines using EtBr, since some cell lines are resistant to this treatment, our results demonstrate that exposure to defined concentrations of EtBr may selectively affect mtDNA, with negligible effects on nDNA using human lymphoblast cell lines derived from CTR individuals and HD patients. However, reversible expression of mtDNA-encoded subunits still precludes the creation of ρ^0 cells derived from CTR and HD human lymphoblasts. These results suggest that the concentrations or the exposure time to EtBr used in this work is not sufficient to deplete mtDNA. Therefore, other concentrations and/or different exposure times are

needed to ensure that reversion rates occur slowly and ρ^0 cells are obtained from lymphoblast cell lines.

-CHAPTER 5. BIBLIOGRAPHY

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