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Human Lymphocytes and *Drosophila melanogaster*¹ as Model System to Study Oxidative Stress in Parkinson's Disease

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1. Introduction

Parkinson's disease (PD, OMIM entry #168600) is the most common progressive neurodegenerative disorder that not only affects a large group of individuals in Antioquia, Colombia (Pradilla et al., 2003; Sanchez et al., 2004) but also affects other regions in the world. Actually, the prevalence of PD is between 0.1% and 0.3% in the general population and between 1% and 2% in persons 65 years of age or older (Alves et al., 2008). Moreover, the number of individuals with PD over age 50 has been projected between 8.7 and 9.3 million in Western countries by 2030 (Dorsey et al., 2007). PD is typified clinically by motor symptoms including bradykinesia, resting tremor, rigidity and gait posture abnormalities followed by postural instability and less frequent non-motor complication such as dementia, depression and autonomic dysfunction (Jancovic, 2008). Pathologically, the disorder is prominently characterized by progressive loss of 50–70% of dopaminergic neurons located in the substantia nigra, decrease of the neurotransmitter dopamine content in striatum (Forno, 1996), cytoplasmic inclusions of insoluble, aggregated proteins, including α -synuclein known as Lewy bodies (Cuervo et al., 2010), elevated levels and/or deposits of iron (Sian-Hülsmann et al., 2010) and selective neuronal vulnerability to oxidative stress (Wang & Michaelis, 2010). The cause of all cases of PD remains unknown. However, in the mid-1990s this situation changed with the identification of a mutation in the α -synuclein gene associated with autosomal dominant PD in Italian kindred (Polymeropoulos et al., 1997). Since then, more than 10 genes have been found either causal of the disease (e.g., *Parkin*, *DJ-1*, PTEN-induced putative kinase 1 (*PINK-1*), leucine rich region kinase 2 (*LRRK2*), *ATP13A2* (Xiomerisiou et al., 2010; Cookson, 2010; Hardy, 2010)) or as risk factor for PD (e.g. HLA region). Interestingly, the first gene that causes autosomal recessive

¹*Drosophila melanogaster* has misleadingly been known as the fruit fly. Strictly, "...real fruit flies,...attack unblemished fruit and in heavy infestations cause serious economic damage. In contrast, even if present in enormous numbers, *D. melanogaster* is innocuous and of no economic importance" (Green, MM. (2002). It really is not a fruit fly, *Genetics* 162: 1-3). It is therefore most adequate to name *Drosophila melanogaster* as just *Drosophila melanogaster* fly

juvenile Parkinsonism (AR-JP) was reported and named *parkin* in 1998 by Kitada and colleagues. AR-JP maps to the long arm of chromosome 6 (6q25.2-q27). The *parkin* gene is composed of 2,960 base pairs with a 1,395-base-pair open reading frame encoding for a protein of 465 amino acids with moderate similarity to ubiquitin at the amino terminus and a RING-finger motif at the carboxy terminus. The gene spans more than 500 kilobases and has 12 exons (Kitada et al., 1998). Subsequent studies have shown that *parkin* is a RING-finger-containing protein identified as an E3 protein-ubiquitin ligase (Shimura et al., 2000), which is an integral component of the cytoplasmic ubiquitin/ proteosomal degradation pathway (Betarbet et al., 2005). The reaction promoted by E3 ligases is the addition of a lysine-linked chain four or more ubiquitin molecules to the target protein, which is recognised by the subunits in the proteasome. Thus, mutation of the *parkin* gene could result in accumulation of misfolded proteins (Tanaka et al., 2001; Imai and Takahashi, 2004). Therefore, it is hypothesized that mutations in *parkin* gene, which result in loss of function, are unable to remove enough mutated or misfolded proteins leading to nigral neurodegeneration. Moreover, the Parkin protein may play a role in promoting autophagy of dysfunctional mitochondria following loss of mitochondrial membrane potential (Bueler, 2010).

Currently, AR-JP (OMIM entry #600116) is considered a distinct genetic entity characterised by early age at onset (<age 45), dystonia with parkinsonism and improvement of symptoms after sleep, slow disease progression, associated signs such as hyperflexia, dysautonomia, peripheral neuropathy and good response to low doses of L-DOPA (Zhang et al., 2001). Additionally, iron deposits are found in PD (Dexter et al., 1989; Sofic et al., 1991; Riederer et al., 1992; Griffiths et al., 1999) as well as in AR-JP (Takanashi et al., 2001). Why dopaminergic neurons in the substantia nigra are particularly vulnerable to the loss of parkin function and iron deposition is yet unknown. To date, the most common known form of hereditary Parkinsonism, i.e. AR-JP, diagnosed in Antioquia, Colombia is due to the *parkin* C212Y mutation. This mutation is a novel G to A transition in exon 6 at position 736 (G736A) of *parkin* gene. The C212Y mutation was identified in a genetic isolate community from two paisa family groups (PJF-1, PJF-3) by Pineda-Trujillo et al., (2001). Interestingly, the mutation was subsequently observed in a Spanish family, suggesting that it could have been taken to Antioquia by Spanish immigrants. Pineda-Trujillo et al., (2006) screened for the G736A mutation in additional Antioquian early onset PD cases and used haplotype analysis to investigate the relationship between Spanish and Antioquian G736A chromosomes. They confirmed the occurrence of an extensive founder effect in Antioquia. Thirteen individuals (10 homozygotes) from seven nuclear families were identified with the G736A mutation. Genealogical investigations demonstrated the existence of shared ancestors between six of these families four to five generations ago and no evidence of Spanish ancestry during this period. A second *parkin* mutation (a duplication of exon 3), was detected in the three G736A heterozygote carriers. Haplotype data exclude a recent common ancestry between the Spanish and Antioquian patients studied and are consistent with the introduction of the G736A mutation in Antioquia during early colonial times by about 16 generations ago. Further studies have also confirmed the presence of a GT insertion in exon 3 mutation among Paisa community previously identified in Spanish and French families with juvenile Parkinsonism (Pineda-Trujillo et al., 2001, 2009). Strikingly, the proteins that are reported to be related to familial PD such as PINK1, DJ-1, α -synuclein, LRRK2 and possibly parkin are either mitochondrial proteins or are associated with mitochondria. Interestingly, all those proteins are involved in pathways that elicit oxidative stress or free radical damage (Lin et al., 2009).

Free radicals are defined as any atom or molecule that has one or more unpaired electrons in its outer shell such as anion superoxide radical ($\cdot\text{O}_2^-$), hydroxyl radical ($\cdot\text{OH}$), nitric oxide ($\text{NO}\cdot$) and their products (e.g. H_2O_2). Oxidative stress (OS) refers to a state in which free radicals are in excess of antioxidant defence mechanism (e. g. superoxide dismutase (SOD), glutathion peroxidase (GPx), catalase, vitamin C and E). As a result of this imbalance, the free radicals are capable of reacting with lipids, proteins, nucleic acids, and other molecules altering their structure and function. Accordingly, OS can lead to serious structural modifications in cells by excessive accumulation of oxidized products such as aldehydes and isoprostanes from lipid peroxidation, protein carbonyls from protein oxidation, and base adducts from DNA oxidation. Because the human brain is a high oxygen consumer organ, it is reasonable to assume that, under pathological conditions, it might be a target of permanent OS attack.

Over the last two decade, OS has been proposed to play a critical role in the pathogenesis of PD (Fahn and Cohen, 1992; Jenner & Olanow, 1996; Tsang & Chung, 2009). In fact, several markers of OS have been identified in post-mortem brain tissues including increased levels of DNA and RNA oxidation (e.g. 8-hydroxyl-2-deoxyguanosine and 8-hydroxyl deoxyguanosine), protein carbonyl levels, glycation and glycooxidation, lipid peroxidation and high iron concentration (Zhou et al., 2008). Moreover, given that iron and DA generate reactive oxygen species (ROS), they have been implicated in the OS observed in PD (Asanuma et al., 2004). Not surprisingly, lymphocytes have been used to test for oxidative stress (Battisti et al., 2008) and cell death (Calopa et al., 2010) in PD. For instance, Migliore et al., (2002) has demonstrated an increase in the incidence of spontaneous micronuclei, single strand breaks and oxidized purine bases in PD patients without treatment. These results clearly showed oxidative DNA damage demonstrable in lymphocytes. Moreover, we found that homozygote Cys212Tyr *parkin* mutation in AR-JP patients renders lymphocytes sensitive to dopamine, iron and hydrogen peroxide stimuli (Jimenez-Del-Rio et al., 2004). In agreement with these findings, Prigione and co-workers (2009) have shown increased oxidative stress in lymphocytes from untreated Parkinson's disease patients. Interestingly, Jiang et al., (2004) have shown that parkin protects human dopaminergic neuroblastoma cells against dopamine-induced cell death. Taken together these data suggest that analysis of DNA or lymphocytes response against oxidative stress might be used as an early marker of the OS status in PD patients.

Despite these evidences, there are still major unresolved issues in the understanding of the molecular and cellular biology of PD. Indeed, a complete picture of the precise molecular cascade leading to cell death in a single cellular model in this disorder is still lacking. Therefore, we have been interested in investigating the oxidative stress phenomenon and apoptosis signalling in lymphocytes and *Drosophila melanogaster*.

2. *In vitro* and *In vivo* models

2.1 Human lymphocytes resemble neuronal cells

The brain and the immune system are involved in functionally relevant cross-talk influencing one another's actions, whose main function is to maintain homeostasis. Therefore, to play such a role, lymphocytes are equipped with several biochemical systems that display comparable pathways to neural cells. This unusual characteristic makes lymphocytes an excellent *in vitro* model (Massaud et al., 1998; Kriesberg, 2011) to understand normal and abnormal function from gene to phenotype. Moreover, lymphocytes

might provide the basis of biochemical and cytopathological mechanisms for preventive or therapeutic intervention. These cells thus appear to be particularly fascinating cell model for PD at least for three main reasons. First, lymphocytes express six homologous neurochemical systems (Table).

System	Protein Expression
1. Dopaminergic	Tyrosine hydroxylase & monoamine oxidase (Marino et al., 1999 & references within); dopamine transporter (Amenta et al., 2001; Marazziti et al., 2010); dopamine D2-, D3-, D4-, D5-like receptors (Ricci & Amenta, 1994; Ricci et al., 1995, 1997; Amenta et al., 1999; McKenna et al., 2002).
2. Serotonergic	Serotonin transporter (SERT, Faraj et al., 1991; Marazziti et al., 2010); serotonin receptors (Stefulj et al, 2000); tryptophan hydroxylase (Carrillo-Vico et al., 2004).
3. Cholinergic	Acetylcholine (Ach), muscarinic and nicotinic Ach receptors (mAChRs and nAChRs), choline acetyltransferase (ChAT), high affinity choline transporter and acetylcholinesterase (Kawashima & Fujii, 2004).
4. Glutamatergic	Ionotropic glutamate receptors (Lombardi et al., 2001, 2004); group I metabotropic glutamate receptors (Miglio et al., 2005).
5. Adrenergic	β -2 adrenergic receptors (Sanders, 1998).
6. Gabaergic	γ -aminobutiric acid (GABA) receptors (Tillakaratne et al., 1995).

Table 1. Neuronal Molecular systems expressed in lymphocytes.

Second, lymphocytes express similar molecular death machinery leading to typical morphologic and biochemical features of apoptosis. Apoptosis is a type of programmed cell death initially defined by Kerr and co-workers in 1972 and recently refined by several others (Kerr et al., 1995; Xu & Shi, 2007; Kroemer et al., 2009). Apoptosis is originally a morphological phenomenon characterised by chromatin condensation and nuclear fragmentation, plasma membrane blebbing, cell shrinkage and preservation of organelles such as mitochondria. These characteristics can be recognised in lymphocytes under fluorescent microscopy (Fig. 1) or electron microscopy (Sakahira et al., 1999; Marini et al., 2001). Noticeably, what causes these morphological changes that we recognize as apoptosis occurs through multiple independent pathways that are initiated either from triggering events within the cell (i.e the “intrinsic pathway”) or from outside the cell (i.e. the “extrinsic pathway”). The “intrinsic pathway” involves the release of mitochondrial proteins such as cytochrome C, second mitochondrial-derived activator of caspase/direct IAP-associated binding protein with low PI (Smac/DIABLO), apoptosis inducing factor (AIF) and Endonuclease G (Endo G). The “extrinsic pathway” involves Fas/FasL pathway, caspase-8 activation, bid degradation and releasing cytochrome C. Strikingly, both pathways converge

on a common machinery of cell dismantling executed by a family of cysteine proteases known as Caspases. Indeed, caspases cleavage at aspartate residues of targeted proteins (Chowdhury et al., 2008). Particularly, caspase-3 degrades the inhibitor of caspase-activated DNase (ICAD/ DNA fragmentation Factor-45, DFF-45) protein releasing the caspase-activated DNase (CAD/ DFF-40) that result in DNA degradation ("DNA ladder pattern") from mouse T-cell lymphoma (Enari et al., 1998; Sakahira et al. 1998), Jurkat T cells (Liu et al, 1997) and HeLa cells (Halenbeck et al., 1998) under pro-apoptotic treatments. It is worth to mention that almost 18-years passed before an explanation could be drawn for one of the earliest well-recognized biochemical characteristics of apoptosis i.e. "DNA ladder pattern", from the time when Wyllie reported glucocorticoid-induced thymocytes apoptosis associated with endonuclease activation (Wyllie, 1980). Unquestionably, morphological and biochemical data have helped considerably to enlighten, yet unsettled, the mechanism of neural cell death in PD (Levy et al., 2009).

Fig. 1. Human lymphocytes treated with xenobiotic paraquat, PQ for 24h. Figure shows typical nuclear apoptotic morphology such as highly condensed chromatin (arrows) and nuclear fragmentation (arrowheads) from lymphocytes treated with PQ compared to normal nuclei (asterisk) stained with acridine orange/ ethidium bromide. A similar apoptotic morphology can be observed with dopamine, DA; 6-hydroxydopamine, 6-OHDA; 5,6 & 5,6 dihydroxydopamine, 5,6 & 5,7-DHT; rotenone, ROT. Jimenez-Del-Rio & Velez-Pardo, 2008. Reproduced with permission from Informa Healthcare UK Ltd.

Third, lymphocytes and neurons are post-mitotic cells, i.e. they become locked in a G_0 phase of the cell cycle. This is a remarkable biological feature to be cautiously considered when interpreting experimental data since evidence has accumulated that a cell division forced on a mature neuron leads to apoptosis rather than division (Herrup et al., 2004), but cell division is induced in lymphocytes. In other words, the use of cell lines instead of primary cultures could be confusing and /or misleading. For instance, NF- κ B is a transcriptional

factor composed of a p50/p65 heterodimer protein that upon activation binds to specific DNA sequences in target genes, designated as κ B-elements. This factor is involved in both cell cycle-regulation and cell death processes. In dividing cells, NF- κ B transcribes cyclin D1, which in association with cyclin-dependent kinases, CDK4 and CDK6, promotes G1/S phase transition through CDK-dependent phosphorylation of retinoblastoma protein (pRb), thereby releasing the transcription factor E2F, required from the activation of S phase-specific genes. Indeed, constitutive activation of NF- κ B is intimately intertwined with cancer growth and metastasis (Prasad et al., 2010). On the other hand, the regulatory roles of NF- κ B on apoptosis suggest that NF- κ B is acting on the upstream pathways of apoptosis, either negatively or positively (Shishodia & Aggarwal, 2004; Qin et al., 2007). Noticeably, in non-dividing cells, these confounding matters connected with the role of NF- κ B in apoptosis and cell-cycle control might not be an important issue given that NF- κ B function can eventually be studied independently from the cell cycle function. Thus, G_0 represents not simply the absence of signals for mitosis but an active repression of the genes needed for mitosis.

2.1.1 Human lymphocytes as cellular model to study oxidative stress and apoptosis in PD.

Deciphering the Parkinson's disease cascade(s) is one of the ultimate research goals in the PD field not only because it offers the possibility to scrutinize a basic cellular machinery of response to different deleterious stimuli, but also because it brings the possibility to predict novel therapies. Accordingly, we postulated a unified molecular cascade model wherein H_2O_2 is definitely a paramount molecule involved in intracellular signalisation that induces neuronal loss in PD (Jimenez-Del-Rio & Velez-Pardo, 2000, 2004a & Fig. 2). Effectively, we were able to clarify the major signalling events by which DA (Jimenez-Del-Rio et al., 2004), monoamine related toxins (e.g. 6-OHDA; 5,6-DHT; 5,7-DHT: Jimenez-Del-Rio & Velez-Pardo, 2002), redox metals such as Fe^{2+} , Cu^{2+} , Mn^{2+} , Zn^{2+} (Jimenez-Del-Rio & Velez-Pardo, 2004b & Fig. 3) and H_2O_2 (Jimenez-Del-Rio & Velez-Pardo, 2006) might induced cell death in normal and/or mutated lymphocytes (e. g. C212Y in parkin) PD.

During the last few years, several reports have been published supporting our findings. Liang et al., (2007) have found that NF- κ B contributes to 6-OHDA-induced apoptosis of nigral dopaminergic neurons through p53. Bernstein and co-worker (2011) have shown that 6-OHDA generated ROS induces DNA damage and p53- and PUMA-dependent cell death. Bilobalide, which is a constituent of *Ginkgo biloba* 761, inhibits 6-OHDA-induced activation of NF- κ B and loss of dopaminergic neurons in rat substantia nigra (Li et al., 2008). Importantly, Aleyasin et al., (2004) have shown that acute inhibition of NF- κ B via expression of a stable I κ B mutant, down-regulation of the p65 NF- κ B subunit by RNA interference (RNAi), or pharmacological NF- κ B inhibitors significantly protected against DNA damage-induced neuronal death. NF- κ B inhibition also reduced p53 transcripts and p53 activity as measured by the p53-inducible messages, Puma and Noxa, implicating the p53 tumor suppressor in the mechanism of NF- κ B-mediated neuronal death. Takada et al. (2003) have shown that H_2O_2 activates NF- κ B through tyrosine phosphorylation of I κ B α and serine phosphorylation of p65 by I κ B α kinase and Syk protein-tyrosine kinase. Prabhakaran et al., (2008) have shown that NF- κ B induction and the activation of nitric oxide synthase through ROS represents a proximate mechanism for Mn-induced neurotoxicity. Therefore, we conclude that NF- κ B, p53 and caspase-3 are crucial signalling molecules involved in H_2O_2 -induced cell death. Based on this model, we predicted that molecules capable of generating

Fig. 2. **Schematic model of dopaminergic and serotonergic related toxins-induced apoptosis by an oxidative stress mechanism in PBL.** 6-OHDA; 5,6-& 5,7-DHT or protein fragment Aβ generate H₂O₂. This last compound might activates JNK/SAPK kinases pathway, which in turn activate in parallel both NF-κB and c-Jun transcription factors. NF-κB is able to activate the transcriptional factor p53 and subsequently it may activate the pro-apoptotic Bax protein, which induces cytochrome C release from mitochondria to activate the apoptosome complex leading to caspase-3 activation and apoptosis. Jimenez Del Rio and Velez-Pardo, 2002. Reproduced with permission from Elsevier.

H₂O₂ might induce a mechanism resembling the one depicted in Fig. 2. To further test our model, we used paraquat (PQ), also known as methyl viologen dichloride or 1,1'-dimethyl-4,4'-bipyridinium dichloride, and rotenone (ROT), a redox cycling herbicide and a mitochondrial complex I inhibitor as xenobiotic compound generally used to model PD (Bové et al., 2005). We concluded that both PQ-and ROT-induced time- and concentration-dependent apoptosis in lymphocytes which was mediated by anion superoxide radicals (O₂•) / hydrogen peroxide, depolarization of mitochondria, caspase-3 activation, concomitantly with the nuclear translocation of transcription factors such as NF-κB, p53, c-Jun and nuclei fragmentation (Fig. 4-5, Jimenez-Del-Rio & Velez-Pardo, 2008; Avila-Gomez

Fig. 3. **Schematic representation of the major molecular events induced by metals in lymphocytes.** Fe^{2+} -metal ions in the presence of molecular dioxygen (1) generate superoxide radicals (2), which dismutate either by enzymatic (e.g., superoxide dismutase, SOD) or spontaneously into H_2O_2 (3). This last compound in turn may react with Fe^{2+} to produce hydroxyl radicals (4) ($\text{OH}^{\cdot}$) by Fenton reaction. Over-production of ($\text{OH}^{\cdot}$) may alter the mitochondria transmembrane potential (5) inducing the liberation of different apoptogenic factors and subsequent activation of caspase-3 (6) resulting in disassembly and fragmentation of nuclear chromatin leading PBL to apoptosis (7). The symbol () represents the inhibition (by indicated compound) of the critical steps of the molecular cascade leading to apoptosis by metal ions. Jimenez-Del-Rio & Velez-Pardo, 2004b. Reproduced with permission from Elsevier.

et al., 2010). Interestingly, Choi et al., (2010) have shown that JNK3 mediates PQ- and ROT-induced dopaminergic neuron death. Remarkably, the cell death routine depicted in Fig. 3 can be reversed by the action of cannabinoids (Jimenez-Del-Rio & Velez-Pardo, 2008), IGF-1 (Avila-Gomez et al., 2010) and glucose (Jimenez-Del-Rio & Velez-Pardo, 2008; Avila-Gomez et al., 2010). These data may provide innovating therapeutic strategies to intervene environmentally or genetically susceptible PD population to oxidative stress.

2.1.2 Alternative therapies for parkinson's diseases: a mechanistic igf-1, cannabinoids and glucose proposal

Based on recent progress in delineating the disease cascade and cell death process (Jenner & Olanow, 1998; Blum et al., 2001; Wirths et al., 2004; Jimenez-Del-Rio & Velez-Pardo, 2004a; Green & Kroemer, 2005; Przedborski, 2005; Jimenez-Del-Rio & Velez-Pardo, 2008; Avila-Gomez et al., 2010), discrete types of potentially disease modifying treatment could be administered for PD. In this regard, our data have highlighted the potential use of lymphocytes as a model to screen antioxidant strategies designed to remove (Fe^{2+})/(O_2)/(H_2O_2)/($\text{OH}^{\cdot}$), signalling inhibitors and/or restorative approaches as promising

Fig. 4. **Schematic model of the major molecular events induced by PQ in lymphocytes.** PQ in the presence of NADH/NADPH reductases (1) is converted into monocationic radical compound which readily react with molecular dioxygen to generate superoxide radicals (2), which dismutase either by enzymatic (e.g. superoxide dismutase, SOD) or spontaneously into H₂O₂ (3) This last compound in turn may activate the mitogen-activated protein kinase kinase kinase (e.g. MEKK1) which can activate both c-Jun (4) via activation of MKK4/JNK, and NF-κB activation (5) via phosphorylation of the IκBa (i.e. the repressor of NF-κB) by the IKK complex. The NF-κB translocates into the nucleus and transcribes p53 protein (6). Consequently, this protein transcribes pro-apoptotic proteins (e.g. Bax) which are able to permeabilize mitochondria, thus, promoting the activation of caspase-3 (7) which signals chromatin fragmentation, typical of apoptotic morphology (8). The symbol () represents the inhibition (by indicated compound) of the critical step of the molecular cascade leading to apoptosis by PQ. Jimenez Del Rio & Velez-Pardo, 2008. Reproduced with permission from Informa Healthcare UK Ltd.

Fig. 5. PQ induces simultaneous activation of the transcription factors in lymphocytes. PBL cells were left untreated (A–C) or exposed to 1mMPQ (D–F) for 24 h. After this time of incubation, cells were stained with anti-NF- κ B-p65 (A and D), anti-p53 (B and E) and anti-c-Jun (C and F) antibodies according to procedure described in Materials and methods. Notice that NF- κ B, p53 and c-Jun positive-nuclei (dark brown color) reflect their nuclear translocation/activation and appear to correlate with the apoptotic nuclear morphology, i.e. condensed/fragmented nuclei when compared with untreated cells (A–C). Magnification 400 $\times$ (A–F). Jimenez-Del-Rio & Velez-Pardo, 2008. Reproduced with permission from Informa Healthcare UK Ltd.

therapy for PD. As depicted in Figs. 2-4, these mechanistic pathways may be of potential use for screening pharmacologically chemical libraries containing hundreds to thousands of compounds each that could modulate or control sensible molecules critical in cell fate (e. g., H_2O_2 , NF- κ B, p53, c-Jun, caspases). Recently, neurotrophic factors have come into focus as potential therapy in PD (Evans et al., 2008). One clue of its neuroprotective capability comes from the fact that IGF-1 is able to activate NF- κ B against H_2O_2 oxidative stress (Heck et al., 1999). However, it has also been shown that NF- κ B activation is involved in H_2O_2 -induced apoptosis (Kutuk & Basaga, 2003). Therefore, the molecular mechanism(s) that explain the dual role of NF- κ B as attenuator or promoter of apoptosis and the IGF-1's molecular mechanism of neuroprotection still remain to be established.

Taken advantage of the fact that human PBL express IGF-1 receptors (Tapson et al., 1988; Kooijman et al., 1992) and IGF-1 appears to be of potential therapeutic use against PD (Quesada et al., 2008), we were interested in the understanding of the molecular events that are thought to be downstream of IGF-1, in relation to the role played by NF- κ B in survival and death-signalisation against PQ, ROT and H_2O_2 in lymphocytes, as a single cell model. We found that (100 nM) IGF-1 protects lymphocytes from (1 mM) PQ, (250 μ M) ROT and (25, 50, 100 μ M) H_2O_2 -induced apoptosis through NF- κ B activation and p53 down regulation involving the phosphoinositide 3-kinase (PI-3K)-dependent pathway. Interestingly, IGF-1, PDTC (a NF- κ B inhibitor) and pifithrin- α (PFT, a p53 inhibitor) were able to protect and rescue lymphocytes pre-exposed to PQ even when the three compounds were added up-to 6 h post-PQ exposure. Overall these observations suggest that survival and rescue of

lymphocytes from PQ and ROT toxicity is determined by p53 inactivation via IGF-1/ PI-3K pathway (Jimenez Del Rio & Velez-Pardo, 2008; Avila-Gomez et al., 2010).

Which molecular mechanism(s) explain the dual role of NF- κ B as an attenuator or promoter of apoptosis? NF- κ B has been reported to activate both pro-apoptotic genes such as p53 transcription factor (Wu & Lozano, 1994; Hellin et al., 1998; Jimenez Del Rio & Velez-Pardo, 2002; Velez-Pardo et al., 2002; Aleyasin et al., 2004), which in turn activates the expression of several genes that directly control or regulate the process of apoptosis such as Bax, which is a pro-apoptotic Bcl-2 protein family (Xiang et al., 1998), and anti-apoptotic genes such as Bcl-2, Bcl-X_L, X-linked inhibitor of apoptosis (Kairisalo et al., 2009). Therefore, one prevailing model proposes that when the molecular ratio of pro-survival (e.g. Bcl-2, Bcl-x_L, Bcl-w) to pro-death Bcl-2 family members (e.g. Bax, Bad, Bak, Bid) is biased towards pro-death Bcl-2 family members either through changes in expression level, localization or activity, the outer mitochondrial membrane becomes permeable to apoptogenic proteins resulting in the activation of a cascade of effector caspases, such as caspase-3, that kill the cells by irreversible proteolysis of critical nuclear and cytoplasmic constituents. In this vein, our data suggest that IGF-1 might promote gene transcription of survival genes via NF- κ B activation (Kane et al., 1999) and suppresses gene transcription of pro-apoptotic proteins through p53 inactivation. How then p53 turn-off could be related with IGF-1 citoprotection? One possible explanation for this phenomenon comes from the work by Ogawara and colleagues (2002) who showed that Akt enhances the ubiquitination-promoting function of Mdm2 (murine double minute) by phosphorylation of S¹⁸⁶, which results in reduction of p53 protein. Furthermore, Feng and colleagues (2004) showed that PKB/ Akt induces phosphorylation of Mdm2 at Ser¹⁶⁶ and Ser¹⁸⁸ resulting in Mdm2 protein stabilization. Based on this information and our data, it is reasonable to assume that p53 is modulated by IGF-1 through PI3K-Akt pathway. In fact, our findings reveal that p53 but not NF- κ B is the critical transcription factor that may possibly balances the expression of pro-death proteins towards intracellular death decision under oxidative noxious stimuli (Lu, 2005). Therefore, an ideal natural or synthetic pharmacological compound would be one that efficiently function as an antioxidant (e.g. 17 β -estradiol (Jimenez-Del-Rio & Velez-Pardo, 2001; vitamin E) and simultaneously act as a survival signalling molecule (e.g. IGF-1). To our surprise, the molecules exhibiting both features might come from the glandular hairs of *Cannabis sativa* or marijuana, actually known as cannabinoids.

2.1.2.1 Cannabinoids

Cannabinoids are a group of C₂₁ terpenophenolic compounds (Elsohly & Slade, 2005), which exert their effects by binding to specific plasma membrane G-protein-couple receptors, termed CB1 (Matsuda et al., 1990) and CB2 (Munro et al., 1993) receptors. Activation of these receptors has been shown to trigger several G_{i/o}-protein-mediated signalling pathways (Turu & Hunyady, 2010). Although, it is currently accepted that CB1 receptors are specially abundant in basal ganglia, hippocampus, cerebellum and cortical structures; and CB2 receptors are restricted to cell types related to the immune function such as spleen macrophages, tonsils, B cells and natural killer cells, monocytes, neutrophils, and T cells (Pazos et al., 2005), it has also been demonstrated the existence of CB2 receptors in purkinje cerebellar neurons (Skaper et al., 1996), microglia (Klegeris et al., 2003), oligodendrocytes (Molina-Holgado et al., 2002) and brainstem neurons (Van Sickle et al., 2005). Moreover, both receptors elicit similar signalling pathways such as inhibition of adenylate cyclase, stimulation of extracellular-signal-regulated kinase (Demuth & Molleman, 2006) and

activation of phosphoinositide 3-kinase/PKB (Gomez Del Pulgar et al., 2000; 2002; Molina-Holgado et al., 2002; Sanchez MG et al., 2003). The physiological significance of these common characteristics is still unknown.

Cannabinoids have been proposed as potential therapeutic agents against PD (García-Arencibia et al., 2009) thanks to their involvement in control of cell death/ survival decision and in neuroprotection (van der Stelt & Di Marzo, 2005). However, the mechanism of both actions by cannabinoids is far from clear. Moreover, cannabinoids have been shown to function as antioxidant compounds via receptor-independent (Hampson et al., 1998; Chen et al., 2000; Marsicano et al., 2002) or receptor-dependent mechanisms (Nagayama et al., 1999; Kim et al., 2005) or both mechanisms (Kaplan et al., 2003). Although CB antagonists (v. gr. SR141716A) have been used to elucidate the neuroprotective mechanism of cannabinoids, they have not been conclusive (see Marsicano et al., 2002 versus Nagayama et al., 1999; Kim et al., 2005). Therefore, the molecular mechanism(s) of cannabinoids effect on cells is a complex and still controversial issue.

Despite intense investigation, the detailed intracellular mechanism(s) involved in cannabinoids survival effect remains to be elucidated. Because CB2 cannabinoid receptor is linked to activation of PI3K (Sanchez MG et al., 2003), and the non-classical cannabinoid (-)-CP55,940 (a CB1 and CB2 agonist) and JWH-015 (a CB2 agonist) are commercially available, we wanted to elucidate the molecular signalling downstream of CB2 receptor linked to the role played by NF- κ B and p53 in survival and death-signalisation against oxidative stress stimuli. We found that both synthetic agonists protect and rescue PBL against A β ₂₅₋₃₅- and PQ-induced apoptosis by receptor-independent and receptor-dependent pathway (Velez-Pardo & Jimenez-Del-Rio, 2006; Jimenez Del Rio & Velez-Pardo, 2008). In agreement with our previous observations with IGF-1, these results suggest that CP55,940 / (JWH-015) protective and rescue effect on PBL from noxious stimuli is determined by p53 inactivation. Recently, we investigated the ability of CP55,940 and JWH-015 to scavenge reactive oxygen species and their effect on mitochondria permeability transition (MPT) in either a mitochondria-free superoxide anion generation system, intact rat brain mitochondria or in sub-mitochondrial particles (SMP) treated with PQ. Oxygen consumption, mitochondrial membrane potential ($\Delta\psi_m$) and MPT were determined as parameters of mitochondrial function. It was found that both cannabinoids effectively attenuate mitochondrial damage against PQ-induced oxidative stress by scavenging anion superoxide radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), maintaining $\Delta\psi_m$ and by avoiding Ca^{2+} -induced mitochondrial swelling (Velez-Pardo et al., 2010). Understanding the mechanistic action of cannabinoids on mitochondria might provide new insights into more effective therapeutic approaches for oxidative stress related disorders (Fig. 6). Further investigation is needed to classify cannabinoids molecules (Padgett, 2005; Thakur et al., 2005) with effective anti-oxidant from those with pro-oxidant actions.

2.1.2.2 Glucose

Glucose is a soluble sugar added to all cell culture media. In fact, glucose entry to the cell is facilitated by glucose transporters (GLUTs 1-13) (Manolescu et al., 2007) and depending on cell type, the amount of glucose in cell culture formulations ranges from 1 g/L (5.5 mM) to as high as 10 g/L (55 mM). This is an important consideration to take into account because the same processes that can affect cells and molecules *in vitro* can occur *in vivo*. Lymphocytes are ideal for learning about glucose metabolism and resistance against oxidative stress for several reasons. First, these cells express GLU-1 and GLU-3 transporter proteins

Fig. 6. **Scheme of proposed cannabinoid mechanism of action against Paraquat-induced mitochondrial oxidative stress.** High mitochondrial membrane potential ($\Delta\Psi_m$) in intact rat brain mitochondria drives PQ compound into the mitochondrial matrix. Once inside, (1) PQ is reduced to the monocation radical $PQ^{\bullet-}$ at complex I in the respiratory chain by electrons donated from NADH. $PQ^{\bullet-}$ reacts rapidly with O_2 to produce superoxide ($O_2^{\bullet-}$), thereby consuming high amount of oxygen. In turn, the ($O_2^{\bullet-}$) is enzymatically dismutated by MnSOD into H_2O_2 . Then, H_2O_2 induces mitochondrial permeability transition pore (MPT) and decreases $\Delta\Psi_m$. Interestingly, when cannabinoids are present (2), they can remove both $O_2^{\bullet-}$ and H_2O_2 thereby blocking further ROS signaling. Most interestingly, cannabinoids inhibit MPT probably through interactions with the cyclosporine A-binding cyclophilin-D protein (black circle). As a result, cannabinoids maintain the MPT-multiprotein complex (MPC) in a close-stated, high ($\Delta\Psi_m$) but O_2 consumption is still high. Taken in conjunction these actions, cannabinoids thus protect mitochondria from further damage. Velez-Pardo et al., 2010. Reproduced with permission from Springer Publishers Ltd.

(Piatkiewicz et al., 2007). Second, glucose metabolism in lymphocytes is a regulated process. Indeed, glucose can enter glycolytic, pentose phosphate and Krebs cycle pathways (Maciver et al., 2008). Therefore, these cells represent a remarkable non-neural cell model to understanding metabolic regulation of apoptosis and cell survival signaling against stressful stimuli.

Previously, we have demonstrated that PQ- and ROT-induce apoptosis in lymphocytes cultured in standard RPMI 1640 culture medium, which contains 11 mM glucose (11G), via a cascade of molecular events involving $O_2^{\bullet-}$ and H_2O_2 , as prime death signals (Jimenez-De-Rio & Velez-Pardo, 2008; Avila-Gomez et al., 2010). Interestingly, by increasing the concentration of glucose to 55 mM (55G) in RPMI 1640 culture medium, it has been shown

that glucose almost completely protected lymphocytes against PQ- and ROT-induced apoptotic cell death (Jimenez-De-Rio & Velez-Pardo, 2008; Avila-Gomez et al., 2010). These data thus suggest that the predominance of PQ- and ROT-induced oxidative stress damage may be adjusted by decreasing or increasing the concentration of glucose in the cell culture media. By using biochemical analysis and pharmacological inhibition, we found that 55G was effective in suppressing rotenone-induced apoptosis in lymphocytes via four acting pathways which involve the pentose phosphate pathway (PPP-II), glutathione pathway, SOD and CAT antioxidant system and PI3-K signalling. Moreover, it is shown for the first time that glucose induced lymphocyte survival by NF- κ B activation and down-regulation of p53 and caspase-3 (Bonilla-Ramirez, L., Jimenez-De-Rio, M. & Velez-Pardo, C. (2011). Unpublished observations). Taken altogether these results suggest that antioxidants (e.g. cannabinoids), growth factors (e.g. IGF-1) and environmental factor (e.g. glucose) might regulate cell death in lymphocytes upon oxidative stress. Unfortunately, lymphocytes as *in vitro* model of PD do not provide information about executive functions (i.e. cognitive process), kinesthesia (i.e. physical movement) and/or diet-related to PD. To further study the effect of xenotoxicity, diet and movement alterations, we therefore turn our attention to *Drosophila melanogaster*.

2.2 *Drosophila melanogaster*: an unexpected invertebrate in scene

During the last few years, *Drosophila melanogaster* has been recognized as a valuable model to study neurodegenerative diseases (Lu, 2009; Hirth, 2010), especially PD (Botella et al., 2009; Guo, 2010; Whitworth, 2011) for three main reasons. First, some genes implicated as causative of PD have at least one homolog in the fly (e.g. *parkin*, *DJ-1*, *PINK*::see <http://superfly.ucsd.edu> for further information). This unique feature has facilitated the functional interpretation of these genes in the human (Park et al., 2009; Bayersdorfer et al., 2010). Second, the expression of PD related genes in *Drosophila* can be performed by using the binary GAL-4-dependent upstream activating sequence (GAL4/UAS) system (Phelps & Brand, 1998), thus providing an excellent tool to express pathological proteins in the fly's brain (e.g. α -synuclein, Feany & Bender, 2000). Third, the dopaminergic system of the fly is well characterised (Mao & Davis, 2009; White et al., 2010). Furthermore, comparable to the human condition, the *Drosophila* DA system is also involved in locomotor control (Riemensperger et al., 2011). Therefore, the similarity between the dopaminergic network, mode of drug action and behaviour in *D. melanogaster* and mammalian systems, has made the fly a very attractive model for anti-parkinsonism drug discovery (Whitworth et al., 2006). Additionally, *Drosophila* offers the power of rapid drug screening (Pendleton et al., 2002a; Faust et al., 2009). Amazingly, a variety of approaches have been used to model Parkinson's-like motor dysfunction in *Drosophila*, including specific genetic alterations (Feany & Bender, 2000; Pendleton et al., 2002b; Wang et al., 2007; Sang et al., 2007); pharmacological inhibition of crucial proteins in the dopamine system (Pendleton et al., 2002 a, b) or pharmacological insult (Coulom et al., 2004; Chaudhuri et al., 2007). Indeed, previous studies have demonstrated that paraquat (PQ) induces selective cell death of dopaminergic neurons (Chaudhuri et al., 2007) through interaction with complex I of the mitochondrial respiratory chain (Cocheme & Murphy, 2008) and oxidative stress (Bonilla et al., 2006). Therefore, on the understanding that the causes of PD are mainly oxidative stress and mitochondrial dysfunction, antioxidants, free radical scavengers, monoamine oxidase inhibitors, iron-chelators, and other such drugs are expected to be used. The study of

antioxidants is becoming one of the most important subjects in PD research. Based on our *in vitro* data, we investigated the effect of cannabinoids and polyphenols, which are defined as a group of chemical substances present in plants, fruits and vegetables characterized by the presence of one or more than one phenol unit per molecule with several hydroxyl groups on aromatic rings, in *Drosophila melanogaster* against PQ-induced oxidative stress.

Recently, we have shown for the first time that CP55,940, a non-selective CB1/CB2 cannabinoid receptor agonist, significantly protects and rescues *Drosophila* against PQ toxicity via a receptor-independent mechanism (Fig. 7). Interestingly, CP55,940 restores the negative geotaxis activity (i.e., climbing capability) of the fly exposed to PQ. Moreover, *Drosophila* fed with (1–200 μ M) SP600125, a specific inhibitor of the stress responsive Jun-N-terminal kinase (JNK) signalling, and 20 mM PQ increased survival percentage and movement function (i.e., climbing capability) when compared to flies only treated with PQ. Taken together our results suggest that exogenous antioxidant cannabinoids can protect against and rescue from locomotor dysfunction in wild type (Canton-S) *Drosophila* exposed to stress stimuli (Jimenez-Del-Rio et al., 2008). Therefore, cannabinoids may offer promising avenues for the design of molecules to prevent, delay, or ameliorate the treatment of population at high risk of suffering Parkinson disease.

Polyphenols are a group of chemical substances found in plants classified according to their chemical structural as (i) phenolic acids such as gallic (GA), caffeic (CA), coumaric (CouA), ferulic acid (FA), propyl gallate (PG); (ii) flavonoids, which are the largest group of polyphenols, and (iii) non-flavonoid polyphenols. Flavonoids involve anthocyanins and anthoxantins. The latter group is divided into flavonols, flavans, flavanols such as epicatechin (EC), epigallocatechin (EGC) and epigallocatechin-3-gallate (EGCG), flavones and isoflavones (D'Archivio et al., 2007). Numerous studies in the past decade have shown that polyphenols have *in vitro* and *in vivo* activity by preventing or reducing the deleterious effects of ROS associated with oxidative stress and neurodegeneration not only because of their strong antioxidant and metal-chelating properties (Sestili et al., 2002; Melidou et al., 2005; Perron & Brumaghim, 2009), but also because of their capability to induce intracellular signalling pathways associated with cell survival and gene expression (Ramassamy, 2006; Zaveri, 2006). We demonstrated for the first time that pure polyphenols GA, FA, CA, CouA, PG, EC, EGC, and EGCG protect, rescue and, most importantly, restore the impaired movement activity (i.e., climbing capability) induced by paraquat in *Drosophila melanogaster* (Fig. 8). We also showed for the first time that high concentrations of iron (e.g. 15 mM FeSO_4) were able to diminish fly survival and movement to a similar extent as (20 mM) paraquat treatment. Moreover, paraquat and iron synergistically affect both survival and locomotor function. Remarkably, propyl gallate and epigallocatechin gallate protected and maintained movement abilities in flies co-treated with paraquat and iron. Our findings indicate that pure polyphenols might be potent neuroprotective agents for the treatment of PD against stressful stimuli (Jimenez-Del-Rio et al., 2010).

It is generally accepted that the causes of PD are mainly oxidative stress, abnormal protein aggregation and mitochondrial dysfunction. Furthermore, substantial evidence suggests diet (Chen et al., 2007) and environmental risk factors such as pesticides (Dick et al., 2007) and heavy metals (Jones & Miller, 2008), in particular iron intake (Logroscino et al., 2008), as causative of PD. However, how genetic and environmental factors are related to the nutritional status of PD patients is still unknown. Moreover, it has not yet been definitively established whether the nutritional status of PD patients might contribute to the

Fig. 7. Protective effect of antioxidants in *Drosophila m.* exposed to paraquat. Female flies were pre-fed with either 1% glucose alone, 0.5 mM CP55,940 or 0.5 mM vitamin E with 1% glucose in dW for 72 h. Then, flies were left untreated (GLU) or treated with 20 mM paraquat (PQ; vit E + PQ; CP + PQ) for 6, 12, 24 and 48 h. (A) Survival rate (%) and (B) locomotion assay were recorded at the indicated time. *p < 0.05, **p < 0.001. Jimenez-Del-Rio et al., 2008. Reproduced with permission from Elsevier.

Fig. 8. Protective effect of polyphenols in *D. melanogaster* exposed to paraquat. (A) Female flies were pre-fed with either 1% glucose alone or with 0.1 mM gallic acid (GA), ferulic acid (FA), caffeic acid (CA), coumaric acid (CouA), propyl gallate (PG), epicatechin (EC), epigallocatechin (EGC), epigallocatechin gallate (EGCG) polyphenols and 0.1 mg/ml Stalevo® with 1% glucose in distilled water (dW) for 72 h. Then, flies were left untreated (U) or treated with 20mM paraquat (PQ) for 24 and 48 h. Survival rate (%) and (B) locomotion assay were recorded at the indicated time. * $p < 0.05$, ** $p < 0.001$. Jimenez-Del-Rio et al., 2010. Reproduced with permission from Elsevier.

development of the disorder. Therefore, we investigated the effect of glucose in *Drosophila melanogaster* under oxidative stress stimuli.

We have shown that female *D. melanogaster* fed acutely with 20mM PQ in high concentration of glucose (e.g. 10%), as the sole energetic source, not only prolonged survival but also the locomotor activity remained unaltered when compared to fly fed with low concentration of glucose (e.g. 1%) and PQ over a period of 24-48 h (Fig. 9). Additionally, we found that polyphenols protect, rescue and restore the impaired movement activity in *Drosophila* induced by 20 mM PQ in 1% glucose for 24 h exposure (Fig. 8). We also showed that high concentrations of iron (e.g. 10-20 mM FeSO_4) were able to diminish fly survival and locomotor activity over a period of 120 h (5 days). Taken together these findings suggest that either glucose or polyphenols might modulate life span and movement capabilities in *D. melanogaster* exposed to PQ and iron in short time frame. Since there is compelling evidence that shows that the pre-clinical period of PD extends at least 20 years before the motor manifestations (Savica et al., 2010), it is necessary to establish a close parallel with the fly to better understand antioxidant therapy approaches over long period of time. Therefore, we studied the life span and locomotor activity (i.e. climbing capability) of *D. melanogaster* chronically exposed to increasing concentrations of PQ and iron alone or in combination upon 1% or 10% glucose feeding regimen for 15 days and determined whether polyphenols such as GA, PG, EC and EGCG affect the life span and locomotor activity of the fly exposed to PQ for 15 days. It is known that protein aggregation is associated to PD (Tan et al., 2009). Interestingly, high expression levels of the transcription GAL4 protein in *D. melanogaster* have been shown to result in reduced life span (Haywood et al., 2002). Therefore, by using *Ddc-GAL4 Drosophila melanogaster* line, we also investigated whether genetically altered *Ddc-GAL4* flies renders them sensitive to PQ-induced oxidative stress and whether glucose and polyphenols might modulate life span and/or locomotor activity in this line of *Drosophila melanogaster*.

Fig. 9. Effect of glucose concentration in *Drosophila m.* exposed to paraquat.

Male (M) and female (F) were either pre-fed with 1, 5 or 10% glucose (GLU) in distilled water for 72 h. Then, flies were treated with 20 mM paraquat (PQ) for 24 and 48 h. Survival rate (%) was recorded at the indicated time. * $p < 0.05$, ** $p < 0.001$. Jimenez-Del-Rio et al., 2008. Reproduced with permission from Elsevier.

We found for the first time that polyphenols exposure prolong life span ($P < 0.05$ by log-rang test) and restore locomotor activity (i.e., climbing capability, $P < 0.05$ by χ^2 test) of *Drosophila melanogaster* chronically exposed to paraquat compared to flies treated with paraquat alone in 1% glucose (Fig. 10). We found that (10%) glucose partially prolongs life span and climbing in *Drosophila* exposed to iron, PQ or in combination, suggesting that both stimuli enhance a movement disorder in a concentration-dependent and temporal-related fashion. Moreover, chronic exposure of (1 mM) PQ/ (0.5 mM) iron synergistically affect both survival and locomotor function independently of the temporal order of the exposure to the toxicants, but the survival is modulated in a concentration and temporal fashion by glucose. This investigation is the first to report that *Ddc-GAL4* transgenic flies chronically fed with polyphenols increase life span ($P < 0.05$ by log-rang test) and enhance movement abilities ($P < 0.05$ by χ^2 test) compared to untreated *Ddc-GAL4* or treated with paraquat in 1% glucose. Our present findings support the notion that *Drosophila melanogaster* might be a suitable model to study genetic, environmental and nutritional factors as causal and/or modulators in the development of PD. Most importantly, according to our model, we have demonstrated for the first time chronic polyphenols exposure as potential therapeutic compounds in the treatment of PD. These findings altogether open new avenues for the screening, testing and development of novel antioxidant drugs against oxidative stress stimuli (Ortega-Arellano et al., 2011).

3. Conclusion

As noted by the Nobel Prize laureate Dr. S. Brenner (2002) "...choosing the right organism for one's research is as important as finding the right problems to work on..." In this regard, human peripheral blood lymphocytes and *Drosophila melanogaster* as model system are well validated and permit totally controlled experiments, are relatively low cost and ease to use, but most importantly, they resemble neuronal cells and clinical manifestation from PD patients, respectively. As any other model (e.g. animal or human tissue and cell lines), their limitation is your removal from the reality of the whole, integrated physiologic system. Despite this drawback, it turns out that their use in complex biologic investigations such as the one presented in this chapter, introduce lymphocytes and *Drosophila* as a unique opportunity to integrate oxidative stress, cell death, cell survival signalling and therapeutic pathways signalling in a single-cell and organism model.

Our present data support the notion that *Drosophila melanogaster* might be a suitable model to study genetic, environmental and nutritional factors as causal and/or modulators in the development of PD. Most importantly, according to our model, we have demonstrated for the first time that acute cannabinoids or chronic polyphenols exposure as potential therapeutic compounds in the treatment of PD.

These findings altogether open new avenues for the screening, testing, monitoring and development of novel antioxidant drugs against oxidative stress stimuli. Furthermore, based on our present findings, we propose that a combined therapy with antioxidant and high energetic agents should provide to pre-clinical genetically individuals at risk to suffer PD a means to delay or to prevent motor symptoms and/or frank PD-ARJP disorders, as those encounter in Antioquia, Colombia (Pineda-Trujillo et al., 2001, 2006, 2009). These data may contribute to a better understanding of the inherent nutritional status, genetic predisposition and environmental agents as causative factors of PD. However, further studies are needed to fully determine target selection and validation, pharmacology, measurement of efficacy

Fig. 10. Survival (A) and locomotor activity (B) of *Drosophila melanogaster* in absence (0, gray bar) or presence of paraquat (1mM) alone (black bar) or in combination of polyphenols (epicatechin (EC, 0.1 mM, red bar) or epigallocatechin gallate, EGCG gallate (0.1 mM, blue bar) in 1% glucose. Female flies (n= 50 per treatment) were treated as described in *Materials and Methods* section. The graphs show that the proportion of survival and climbing performance dramatically increased in flies exposed to polyphenols compared to PQ treatment alone. Statistical comparisons between treated flies with PQ and polyphenols and PQ alone showed (A) a $P<0.001$ by log-rank test and (B) a $P<0.05$ by χ^2 test. Ortega-Arellano et al., 2011. Reproduced with permission from Elsevier.

(Kieburtz & Ravina, 2007) and bioavailability (D'Archivio et al., 2010) of potential antioxidant molecules, particularly cannabinoids and polyphenols, before one can envision a preventive and effective neuroprotectant therapy against PD.

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Parkinson's disease (PD) results primarily from the death of dopaminergic neurons in the substantia nigra. Current PD medications treat symptoms; none halt or retard dopaminergic neuron degeneration. The main obstacle to developing neuroprotective therapies is a limited understanding of the key molecular mechanisms that provoke neurodegeneration. The discovery of PD genes has led to the hypothesis that misfolding of proteins and dysfunction of the ubiquitin-proteasome pathway are pivotal to PD pathogenesis. Previously implicated culprits in PD neurodegeneration, mitochondrial dysfunction, and oxidative stress may also act in part by causing the accumulation of misfolded proteins, in addition to producing other deleterious events in dopaminergic neurons. Neurotoxin-based models have been important in elucidating the molecular cascade of cell death in dopaminergic neurons. PD models based on the manipulation of PD genes should prove valuable in elucidating important aspects of the disease, such as selective vulnerability of substantia nigra dopaminergic neurons to the degenerative process.

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