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Paraquat, Between Apoptosis and Autophagy

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1. Introduction

Paraquat (PQ, methyl viologen), 1,1'-dimethyl-4,4'-bipyridinium (Figure 1), is a commonly used, potent herbicide. It was first synthesised in 1882 by Weidel and Russo, as recorded by Hadley in his review of 1979 (Haley, 1979), and its redox properties were discovered by Michaelis and Hill in 1933 (Haley, 1979). Initially, PQ was used as an indicator of oxidation-reduction because in the absence of molecular oxygen, donating an electron to paraquat (PQ^{2+}) generated a monocationically stable violet or blue form that is commonly known as methyl viologen (Dinis-Oliveira *et al.*, 2008). However, its properties as an herbicide were not discovered until 1955, and in 1962, it was introduced into global markets.

The PQ is registered and used in approximately 100 countries worldwide and is the second most commonly used herbicide in the world after gliphosate. Despite this, its use is currently banned in the European Union (EU), but the import of products from outside the EU for patients who have been treated with PQ has not.

In its recommended rating of "pesticides by risk," WHO (World Health Organization) considers composite PQ to be moderately toxic (Category II) (World Health Organization 2004). The ECB (European Chemicals Bureau) classifies PQ as being very toxic (R26) by inhalation, toxic (R25) orally and moderately toxic (R24) dermally.

PQ is included in the family of herbicides called bipyridines. It is an herbicide that is non-selective and functions systemically through contact without acting on the leaves of green plants. Among its advantages, it is rapidly absorbed by the leaves of plants that have been sprayed, but clay soil causes it to be biologically inactive.

Its action on plants has been shown to occur on chloroplasts and is based on its redox cycle. PQ interferes with photosynthesis at the level of photosystem I. At this point, PQ blocks the flow of electrons from ferredoxin and $NADP^+$ so that electrons from photosystem I would reduce PQ, which transfers divalent cations (normal state) to monovalent cations (reduced state). The monovalent cation reduces oxygen to the superoxide radical (O_2^-), which is produced by the loss of activity of the chloroplasts and the subsequent cell damage that leads to plant death. There is controversy about the use of PQ in agriculture because herbicides are toxic to humans and the environment, especially when not taking the proper precautions. Specifically, in addition to the adverse effects on humans, one of the greatest

risks occurs in the absorption of the herbicide when being applied to crops. When rats ingested toxic amounts of PQ (either accidentally or voluntary), the initial absorption occurred in the small intestine where the amount absorbed by the stomach was negligible, especially if there was parallel food intake, and the majority was excreted in the urine and feces (Daniel & Gage, 1966). It can also be absorbed and causes damage when it contacts with the skin, especially when there was a previously damaged area that would cause an abrasion contact zone (J. G. Smith, 1988). When applied with a nasal spray, droplets can penetrate the lungs through inhalation. When used in the absence of any physical barrier protection (goggles, masks, gloves, etc.), PQ can be highly toxic. Once absorbed into the body, PQ could affect different organs, with the liver and kidneys being more sensitive to oral ingestion and the lungs being more sensitive to inhalation. In autopsies of dead patients that suffered from voluntary PQ poisoning, different organs were damaged. The brain damage consisted of widespread edema, subepidermal and subarachnoid haemorrhage (which had an uneven distribution in different patients) and inflammation of the meninges, which could be a secondary consequence that resulted from lung damage and hypoxia based on its characteristics (Grant *et al.*, 1980).

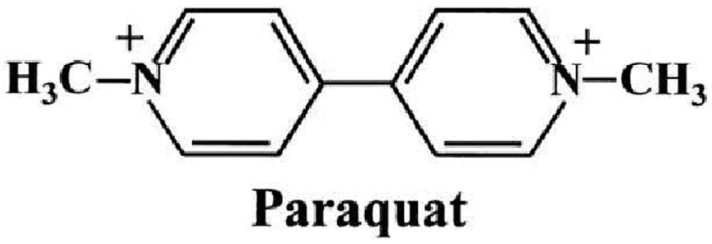

Fig. 1. Chemical structure of paraquat

Together with the correlations observed in epidemiological studies between the use of PQ and the development of Parkinson's disease (PD) (Tanner *et al.*, 2011), the structural similarity between PQ and the active metabolite (MPP^+) of the neurotoxin called MPTP, widely accepted as a model of parkinsonism, led us to postulate the existence of a relationship between the pesticide and the origin of the disease (Costello *et al.*, 2009; Di Monte *et al.*, 1986; Hertzman *et al.*, 1990; Liou *et al.*, 1997). Both neurotoxic effects that generated oxidative stress activated different pathways (Richardson *et al.*, 2005). Currently, PQ is a valid model for studying neurotoxicity based on oxidative stress, such as for MPP^+. Further, studies have examined the relationship between the application and exposure of this pesticide and the development of PD, which is widely accepted for MPP^+, and increasing studies have found a role of PQ in oxidative stress and cell death. The toxicity induced by PQ as an herbicide makes it toxic to mammalian cells. The redox cycling of PQ (Figure 2) in biological systems has two important implications: one is the generation of reactive oxygen species (ROS), and the other is the depletion of reducing agents (NADH and NADPH) necessary for proper function, affecting different cellular processes, such as the synthesis of fatty acids. Similar to inside the plant cell, PQ requires an electron donor to be reduced in neurons. The potential standard reduction (E) of a compound indicates the affinity of the compound to accept electrons. PQ has an E of -0.45 V. The potential E of the

redox couples, NAD^+/ NADH and $NADP^+$/ NADPH, is -0.32V and -0.324V, respectively, where PQ, under physiological conditions and with the aid of diaphorase within the cell, could accept electrons from either reducing agent. The MPP^+ E is - 1.18 V, and this indicates that PQ has a greater ability to accept electrons than MPP^+ (Drechsel & Patel, 2008). Among the cellular enzymes that could donate electrons to PQ (PQ-enzymes with diaphorase), it has been examined mitochondrial complex I (NADH-ubiquinone reductase complex) (Fukushima *et al*., 1993), thioredoxin reductase (Gray *et al*., 2007), NADPH, ferredoxin oxidoreductase (Liochev *et al*., 1994), NADPH oxidase (Bonneh-Barkay *et al*., 2005) and NOS (nitric oxide synthase) (Patel *et al*., 1996) in addition to other enzymes. The mitochondria have been shown to be a major source of ROS generation within the PQ-induced mechanism, which may induce PQ-diaphorase activity during breathing (Drechsel & Patel, 2008). Once PQ has been reduced, it could be oxidised by oxygen and generate superoxide molecules, which occurs in the cell during oxidative stress. This could be activated by different pathways to initiate cell damage through different components and the activation of different cellular mechanisms, such as autophagy (R. A. Gonzalez-Polo *et al*., 2007b), dysfunction of the proteasome (Yang & Tiffany-Castiglioni, 2007) and cell death by apoptosis (Dinis-Oliveira *et al*., 2008; R. A. Gonzalez-Polo *et al*., 2007a; R. A. Gonzalez-Polo *et al*., 2004; McCarthy *et al*., 2004; Niso-Santano *et al*., 2011; Niso-Santano *et al*., 2010; Richardson *et al*., 2005). It is commonly accepted that the key mechanism in PQ-mediated toxicity was due to the oxidative stress-derived superoxide anion produced in the redox cycle (Drechsel & Patel, 2008; Patel *et al*., 1996). The fact that the key element in PQ-mediated toxicity was the generation of superoxide anions was demonstrated by the overexpression or silencing of superoxide dismutase (SOD), which led to an alteration of the toxic effects generated by PQ (Patel *et al*., 1996). The superoxide anion generated in the redox cycling of PQ could be transformed by various reactions and ROS (Bus & Gibson, 1984) primarily generated by the hydroxyl radical (HO^-) and hydrogen peroxide (H_2O_2). These reactive oxygen species have been shown to be responsible for the oxidative stress that initiates different cascades inside the cell and causes apoptosis. Moreover, both cell death and changes in its regulation have been implicated in various diseases, including cancer and neurodegenerative diseases (Leist & Jaattela, 2001). More specifically, various studies have linked apoptosis induced following the exposure to various toxic compounds with the loss of neurons that occur during the development of various neurodegenerative diseases, such as Alzheimer's disease (AD) (Loo et al., 1993) and PD (Andersen, 2001; Fall & Bennett, 1999; Hartmann *et al*., 2000). In addition, previous studies have shown that PQ induced apoptosis in the primary cultures of rat cerebellar granule cells (R. A. Gonzalez-Polo *et al*., 2004) and an increase in the expression of genes related to apoptosis in SH-SY5Y cells (Moran *et al*., 2008). Therefore, we examined PQ as a model for studying the neurotoxicity based on the generation of oxidative stress, such as in PD, to determine the fundamental role of superoxide anions in the redox cycling of the herbicide.

2. Paraquat induces apoptosis

The first time that the term apoptosis appeared was in a paper from John Kerr, Andrew Wyllie and Alastair Currie, in 1972 (Kerr *et al*., 1972). The name was derived from the 'dropping off' or 'falling off' of petals from flowers or leaves from trees.

Apoptosis, or programmed cell death, is characterised by several morphological features, such as DNA degradation into oligonucleosomal fragments, chromatin condensation, reduction in nuclear and cellular fractions, phosphatidylserine exposure on the outward-facing side of the plasma membrane and preservation of organelle structure and plasma membrane integrity, which leads to the generation of apoptotic bodies, or vesicles in the cytoplasm containing tightly packed organelles with or without nuclear fragments. This type of cell death contrasts with necrosis, which is uncontrolled, accidental and pathological cell death. However, these two cell death pathways have been shown to crosstalk, which has been described as the "apoptosis-necrosis continuum" (Zeiss, 2003).

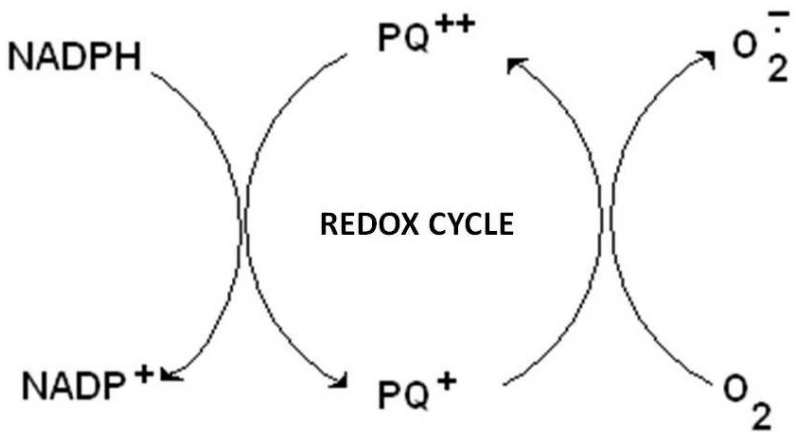

Fig. 2. Redox cycle of paraquat

Previous studies demonstrating the regulation of apoptosis came from *Caenorhabditis elegans* where 131 of the 1090 somatic cells in *C. elegans* were under the control of programmed cell death. In 1985, the Horvitz's lab discovered four important genes (*Ced-3, Ced-4, Ced-9, Egl-1*) that are involved in the regulation of cell death (Fixsen *et al.*, 1985). Previous studies have shown that apoptosis was necessary to define whether cells should live or die. However, there are other forms of programmed cell death and other possible mechanisms that have not yet been discovered (Debnath *et al.*, 2005; Formigli *et al.*, 2000; Sperandio *et al.*, 2000). Several mechanisms of cell death have already been shown to be involved in maintaining the balance between life and death (Boya *et al.*, 2005; Lum *et al.*, 2005; Ravikumar *et al.*, 2006). Apoptosis most commonly occurs during development and aging as a homeostatic mechanism. Although it has been shown to be used as a defence mechanism, such as when cells become damaged, during an immune response (Norbury & Hickson, 2001), or as a pathological process in cancer and autoimmune lymphoproliferative syndrome in which apoptosis was suppressed and led to the development and progression of tumours (Kerr *et al.*, 1994; Worth *et al.*, 2006), neurodegenerative diseases, autoimmune diseases and ischaemia-associated injury where there is excessive apoptosis (Ethell & Buhler, 2003; Freude *et al.*, 2000; C. J. Li *et al.*, 1995). Traditionally, apoptosis has been considered to be an irreversible process; however, several reports have demonstrated that these apoptotic cells could be rescued from programmed cell death (Geske *et al.*, 2001; Hoeppner *et al.*, 2001; Reddien *et al.*, 2001).

The mechanisms of apoptosis are highly complex and involve an energy-dependent cascade of molecular events. Apoptosis can be initiated by a variety of stimuli, but previous studies have shown that there are two main apoptotic pathways: the death receptor or extrinsic pathway and the mitochondrial or intrinsic pathway. However, these two pathways are connected, and some molecules of one pathway have been shown to influence the other pathway (Igney & Krammer, 2002). Further, a third pathway was found in T cells and had been shown to occur through cytotoxicity and perforin-granzyme-dependent cell death (via granzyme A or granzyme B). Basically, the extrinsic pathway is activated by the death receptors (DR), which are localised on the cell surface, through the recognition of their specific ligands. In comparison, the intrinsic pathway is initiated after several intracellular triggers, called "stress signals", such as cytoskeleton disruption, hypoxia, DNA damage, macromolecular synthesis inhibition and endoplasmic reticulum stress, which induce the mitochondria to release pro-apoptotic factors into the cytosol. However, both pathways and the granzyme B pathway terminate in the execution pathway that activates caspases (cysteine-aspartic acid proteases), and they have been shown to be responsible for developing the well-known features of apoptosis (Amarante-Mendes & Green, 1999). In contrast, the granzyme A pathway is a caspase-independent cell death pathway, which has been shown to act in parallel (Martinvalet *et al.*, 2005). The cell's decision has been shown to be determined by the Bcl-2 protein family. The regulation of the Bcl-2 family of proteins is important (Gross *et al.*, 1999).

It is well known that the toxicity of PQ was due to the production of ROS (Bus & Gibson, 1984; Mollace *et al.*, 2003), which has been partially generated by xanthine oxidase (Kitazawa *et al.*, 1991; Sakai *et al.*, 1995). Therefore, it has been shown that PQ could induce apoptotic cell death in cerebellar granule cells using this xanthine oxidase system (R. A. Gonzalez-Polo *et al.*, 2004). In addition, it has been reported that PQ induced apoptosis in other animal models, such as human lung epithelial cells (Cappelletti *et al.*, 1998), PC12 cells (X. Li & Sun, 1999), mouse 32D cells (Fabisiak *et al.*, 1997), primary mesencephalic cells and dopaminergic neuronal cells (Gomez-Sanchez *et al.*, 2010; Peng *et al.*, 2004).

PQ has structural homology to MPP^+, which has been linked to PD in epidemiological studies (Hertzman *et al.*, 1990; Liou *et al.*, 1997). In this vein, *PINK1*-silenced neuroblastoma cells were more sensitive and exhibited increased apoptosis compared with control cells following PQ treatment (Gegg *et al.*, 2009). Silencing *DJ-1* in neuroblastoma cells induced apoptotic cell death, and the treatment with PQ increased apoptosis (R. Gonzalez-Polo *et al.*, 2009).

The cytotoxic actions of PQ have been shown to involve oxidative stress by producing superoxide anions through the mitochondrial electron transport chain (Dinis-Oliveira *et al.*, 2006; McCormack *et al.*, 2002). PQ has been shown to be reduced by mitochondrial complex I and, thus, impair the respiration complex that led to the generation of ROS to induce selective neurodegeneration in dopaminergic neurons in the substantia nigra pars compacta (Fei *et al.*, 2008) and apoptosis by activating different intracellular pathways. PQ has been shown to induce apoptosis through the mitochondrial intrinsic pathway associated with p53 (Yang & Tiffany-Castiglioni, 2008). JNK proteins have been implicated in dopaminergic neuronal death induced by rotenone, PQ and 6-hydroxydopamine (6-OHDA) (Choi *et al.*, 1999; Klintworth *et al.*, 2007; Newhouse *et al.*, 2004; Niso-Santano *et al.*, 2006). PQ activates cell death through JNK and its downstream target c-Jun (Peng et al. 2004) and induces high levels of pro-apoptotic Bcl-2 family members (Bak, Bid, BNip3 and Noxa) in conjunction with cytochrome c release and caspase-3 activation (Fei *et al.*, 2008).

Another mechanism by which PQ has been shown to activate cell death involves chronic endoplasmic reticulum (ER) stress (Chinta *et al.*, 2008; Holtz & O'Malley, 2003; Ryu *et al.*, 2002). The increase in the expression of GRP family proteins, the increased phosphorylation of eIF2a and the induction of GADD153 expression was reported following PQ treatment in dopaminergic N27 cells (Chinta *et al.*, 2008) . These results were consistent with previous studies that demonstrated the transcriptional upregulation of ER stress and unfolded protein response (UPR)-specific proapoptotic genes following exposure to MPP^+ and 6-OHDA (Holtz & O'Malley, 2003; Ryu *et al.*, 2002). Several neurodegenerative diseases feature the accumulation of abnormal proteins as a result of the inhibition of the cellular proteasome activity and ER stress. Paraquat treatment led to a significant decrease in 20S proteasome activity (Chinta *et al.*, 2008). The inhibition of proteasome activity initiated the formation and accumulation of ubiquitinated protein aggregates (Lam *et al.*, 2002).
PQ was shown to induce IRE1/ASK1/JNK activation (Niso-Santano *et al.*, 2010; Yang *et al.*, 2009). IRE1 is an ER-resident transmembrane protein that is activated in response to ER stress. IRE1 phosphorylates ASK1, which has been shown to play a key role in the activation of p38/JNK signalling in neurotoxin-induced cell culture models of PD, such as MPTP and paraquat-induced apoptosis in dopaminergic neuronal cells (Niso-Santano *et al.*, 2010).
Recent studies have shown that paraquat induced acetylation of core histones in cell culture models of PD and that the inhibition of HAT activity by anacardic acid significantly attenuated paraquat-induced caspase-3 enzyme activity, indicating that histone acetylation played a role in paraquat-induced apoptosis (Song *et al.*).

3. PQ induces autophagy

Autophagy is an intracellular lysosome-mediated catabolic mechanism that is responsible for the bulk degradation and recycling of damaged or dysfunctional cytoplasmic components and intracellular organelles (Klionsky & Emr, 2000). Autophagy is an evolutionarily conserved cellular response to both extracellular stress conditions (nutrient deprivation and hypoxia) and intracellular stress conditions (accumulation of damaged organelles and cytoplasmic components). Autophagy is a physiological degradative process employed during embryonic growth and development, cellular remodelling and the biogenesis of some subcellular organelles, such as multi-lamellar bodies (Filonova *et al.*, 2000; Hariri *et al.*, 2000; Sattler & Mayer, 2000). Autophagic cell death has been shown to involve the accumulation of autophagic vacuoles in the cytoplasm of dying cells and in mitochondrial dilation and the enlargement of the ER and the Golgi apparatus.
Different types of autophagy are classified depending on the mechanism driving the degradation of the substrate in the lysosomal lumen (Klionsky *et al.*, 2007). We could distinguish three types of autophagy:
1. Macroautophagy: is often referred to as "autophagy". In this process, the material to be degraded becomes trapped in double-membrane vesicles to form a structure known as the autophagosome (Baba *et al.*, 1994; Fengsrud *et al.*, 1995). Macroautophagy has been shown to involve a number of genes called *ATGs* (autophagy-related genes), which have been shown to encode more than 30 proteins. Autophagosome membranes are derived from a structure called the pre-autophagosome, phagophore or early autophagosome (Fengsrud *et al.*, 1995; Mizushima *et al.*, 2001; Suzuki *et al.*, 2001). The first step towards the formation of the late autophagosome is the expansion of the phagophore (pre-autophagosome) membrane. Therefore, the carbon terminus of the protein LC3 (encoded by the gene *ATG8*) is attached to

a residue of phosphatidyl-ethanolamine (PE) in the membrane of the phagophore and with two other proteins encoded by *ATG12* and *ATG5* that also bind to the inner membrane, leading to the formation of the autophagosome. This autophagosome then fuses with lysosomes, forming the autophagolysosome where the degradation of the material occurs due to the action of lysosomal enzymes.

2. Microautophagy: In this process, the material to be degraded becomes trapped by the lysosomes through the invagination of its membrane. Once introduced into the lysosome, the material becomes degraded by lysosomal enzymes similar to macroautophagy.

3. Chaperone-mediated autophagy (CMA): In this autophagy, the material to be degraded is damaged or misfolded protein that has been translocated into the lysosomal lumen through the lysosomal membrane. This translocation is mediated by cytosolic and lysosomal chaperones, involving the carrier LAMP-2A (lysosome-associated membrane protein 2A).

Dysfunctions in autophagy have been implicated in various diseases, such as cancer (Kondo & Kondo, 2006), cardiomyopathy (Nakai *et al.*, 2007) or neurodegenerative processes (Martinez-Vicente & Cuervo, 2007; Ravikumar & Rubinsztein, 2004). In neurodegenerative diseases, an increase in the formation of autophagic vacuoles in the *substantia nigra* of patients with PD (Anglade *et al.*, 1997), Huntington's disease (Kegel *et al.*, 2000; Sapp *et al.*, 1997) and AD (Butler & Bahr, 2006; Nixon *et al.*, 2005; Zheng *et al.*, 2006) has been shown. This raises questions about the role of autophagy in these neurodegenerative processes. Previous studies have suggested that the increased number of autophagic vacuoles was responsible for neuronal death; however, in contrast, other studies have suggested a protective role for autophagy, contributing to the increased degradation of damaged proteins, which could induce apoptosis (U. Bandyopadhyay & Cuervo, 2007).

Changes have been described in the ubiquitin-proteasome system associated with PD. Several studies have suggested that once the ubiquitin-proteasome system has been damaged, autophagy becomes over-regulated, increasing the number of protein aggregates degraded by this mechanism (Iwata *et al.*, 2005; Massey *et al.*, 2006), which has been considered to be the default pathway when protein aggregates could not be eliminated by the proteasome (Olanow, 2007; Rideout *et al.*, 2004). However, if the pathogenic insult was maintained, this compensatory mechanism was unable to maintain cellular balance, leading to neuronal death (Trojanowski & Lee, 2000).

Recently, converging evidence suggests that the impairment of homeostatic mechanisms processing unwanted and misfolded proteins plays a central role in the pathogenesis in PD (Olanow, 2007). Impairment of the autophagy-lysosomal pathway has been shown to be related to the development of PD (Pan *et al.*, 2008). Activation of autophagy was also identified within peripheral blood mononuclear cells from PD patients (Prigione *et al.*, 2010). This self-regulatory concept of autophagy supports the hypothesis that increased signalling of autophagy occurred in mice with a malfunctioning lysosome that was accompanied by the aggregation of the protein α-synuclein (Meredith *et al.*, 2002). Increased levels of α-synuclein were reported in both the frontal cortex and the ventral midbrain, and α-synuclein positive inclusions in the substantia nigra neurons of mice treated with PQ were found (Manning-Bog *et al.*, 2002). The association of dopaminergic neuronal death with α-synuclein upregulation and aggregation following PQ toxicity is relevant as a PD model.

Because PQ induced the accumulation of autophagic vacuoles and increased the degradation of proteins in the cytoplasm of SH-SY5Y cells (R. A. Gonzalez-Polo *et al.*, 2007a), this indicates that the increased oxidative stress could activate autophagy in the initial stages of mitochondrial dysfunction to have a protective role in paraquat-induced cell death

(R. A. Gonzalez-Polo *et al.*, 2007a, 2007b). Moreover, our group has shown that PQ exposure induced an early reticulum stress response that was correlated with the adaptive activation of autophagy, characterised by the accumulation of autophagic vacuoles, activation of beclin-1, accumulation of LC3-II, p62 degradation, and mammalian target of rapamycin dephosphorylation (R. A. Gonzalez-Polo *et al.*, 2007a, 2007b; Niso-Santano *et al.*, 2011). This response was increased in cells that overexpressed wild-type (WT) ASK1 (apoptosis signal kinase 1) protein. In this model, the inhibition of autophagy caused an exacerbation of the apoptosis induced by ASK1 WT overexpression with or without PQ. These results suggest that autophagy has an important role in the cell death/survival events produced by PQ and ASK1 that contribute to neuronal degeneration.

Therefore, increased autophagy might be a new strategy for the treatment of neurodegenerative diseases (Menzies *et al.*, 2006). It is encouraging to consider enhancing the autophagic capacity as a therapeutic strategy in the prevention of neurodegeneration because studies have shown that the abnormal regulation of autophagic pathways may lead to apoptosis and cell death (Walls *et al.*, 2010).

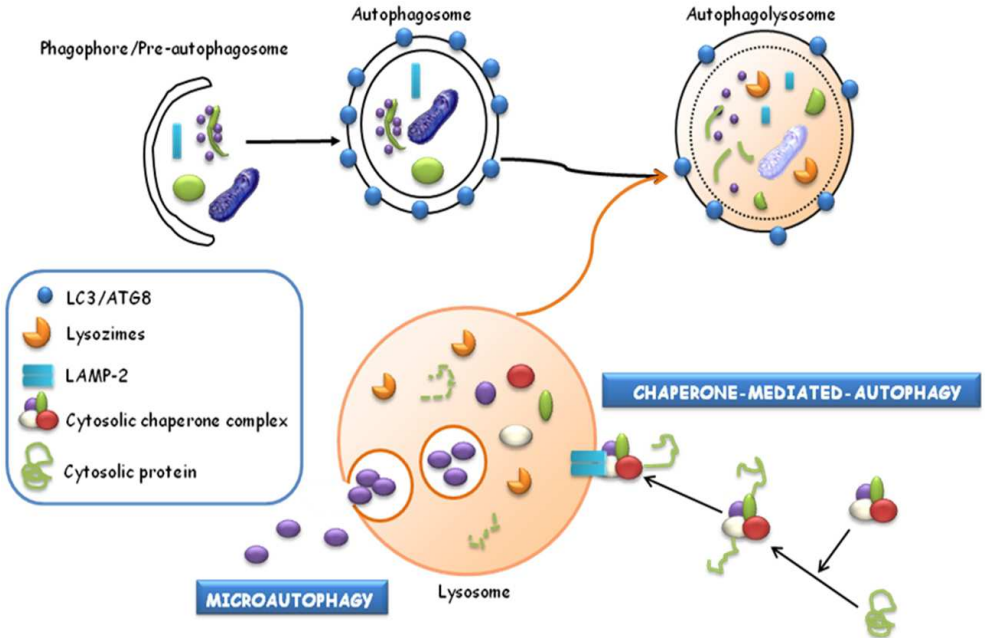

Fig. 3. Schematic representation of the different types of autophagy

4. Paraquat and Parkinson´s disease-related proteins

As previously described, PD is characterised by the selective degeneration of dopaminergic neurons. The aetiology of PD is unknown but has a multifactorial origin that involves both genetic and environmental factors. The interaction of both factors was, in part, involved in the selective death of dopaminergic neurons observed in PD. Apart from the studies that have identified human mutations as a basis for disease, the high number of individuals with

sporadic PD have an unknown aetiology. These individuals have multifactorial disease in which the environment plays important roles. PQ is an environmental agent that has been associated with PD. A recent study by Caroline Tanner concluded that people using PQ and rotenone were 2.5 times more likely to develop PD than those who were not in contact with them (Tanner *et al.*, 2011). Therefore, there is a relationship between the toxicity of PQ and PD. This interaction is not known; however, several studies directly indicate the interaction of PQ with PARK genes.

The development of PD was attributed to different events, such as mitochondrial dysfunction, oxidative stress or the aggregation of proteins. These events could be important to understanding the relationship between PQ and PARK genes (Table 1).

Gene	Locus	Protein name	Inheritance	Function
PARK 1/4	4q21.3-q22	α-synuclein	AD	Lewy`s body component
PARK 2	6q25-27	Parkin	AR	E3 ubiquitin ligase
PARK 3	2p13	¿?	AD	¿?
PARK 5	4p14	UCHL-1	AD	Ubiquitin C-terminal hydrolase
PARK 6	1p35-36	PINK1	AR	Mitochondrial kinase
PARK 7	1p36	DJ-1	AR	Antioxidant agent
PARK 8	12q12	LRRK2	AD	Kinase, GTPase
PARK 9	1p36	ATP13A2	AR	ATPase, cationic transport
PARK 10	1p32	¿?	AD	¿?
PARK 11	2q36-q37	GIGYF2	AD	Receptor tyrosine kinase signaling
PARK 12	Xq21-q25	¿?	X-linked	¿?
PARK 13	2p13	HTRA2/OMI	AD	Serine protease
PARK 14	22q13.1	PLA2G6	AR	Fosfolipase A2
PARK 15	22q11.2	FBXO7	AR	E3 ubiquitin ligase
PARK 16	1q32	RAB7L1	¿?	¿?
PARK 17	4p	GAK/DGKQ	¿?	¿?
PARK 18	6p	HLA-DRA	¿?	¿?

Table 1. Some characteristics of PARK genes

The increase in oxidative stress has been observed in the substantia nigra of PD brains, as demonstrated by the increased lipid, protein, and DNA oxidation or increased total iron content (Bagchi *et al.*, 1995; Mattson, 2006). This alteration of cellular redox balance may be produced by different mechanisms because of the enzymatic conversion to secondary reactive products and/or ROS by the depletion of antioxidant defences or the impairment of antioxidant enzyme function (Abdollahi *et al.*, 2004). Autosomal recessive PD-associated genes such as parkin, DJ-1 and PTEN-induced putative kinase 1 (PINK), have been shown to be involved in mitochondrial function, which suggests that mitochondrial dysfunction and the generation of ROS were central events in the pathogenesis of PD. Therefore, further study of the implication of these proteins in PQ toxicity would be of interest.

In contrast, the misfolding and aggregation of proteins is another pathway of cell toxicity in PD. The failure of α-synuclein (PD-related protein) clearance by the ubiquitin-proteasome system UPS (ubiquitin proteosome system) led to its accumulation over time and to the formation of fibrillar aggregates and Lewy bodies. In this vein, there is a relationship between PQ toxicity and PD because exposure to PQ has been shown to induce proteasome dysfunction and α-synuclein aggregation (Ding & Keller, 2001; Fei *et al.*, 2008; Goers *et al.*, 2003; Manning-Bog *et al.*, 2002; Yang & Tiffany-Castiglioni, 2007).
Therefore, there is a relationship between the toxicity exerted by PQ and different *PARK* genes.

4.1 α-synuclein (PARK1, PARK4) and paraquat

Lewy bodies (LBs) are abnormal aggregates of protein that develop inside the nerve cells in PD. The presence of α-synuclein in these aggregates has been shown to play an important role in the formation of LBs (Masliah *et al.*, 2000; Spillantini *et al.*, 1997). The mechanisms that promote intraneuronal α-synuclein assembly remain poorly understood. Missense mutations (A53T, A30P and E46K) or multiplications (duplications and triplications) in the *a-synuclein* gene (*PARK1/4*) caused autosomal-dominant parkinsonism (Polymeropoulos *et al.*, 1997), but it is still unclear whether fibrils of aggregated α-synuclein, as found in LBs, have a causative role in the more common forms of PD or could be a marker for the underlying pathogenetic process. α-synuclein has three common forms, monomers, dimers, and protofibrils, and it is thought that an excess of the protofibril forms inhibited UPS *in vitro* (McNaught *et al.*, 2001) and *in vivo* (Dyllick-Brenzinger *et al.*, 2010). α-synuclein protofibrils have been shown to directly lead to oxidative stress that could further impair UPS by reducing ATP levels, inhibiting the proteasome and by the oxidation of parkin.
Studies have indicated that the interaction of environmental factors with alterations in α-synuclein might be involved in the aetiology of PD. The interaction of α-synuclein with PQ toxicity has been extensively examined. PQ has been shown to potentiate α-synuclein-induced toxicity (Norris *et al.*, 2007). PQ preferentially binds to the partially folded α-synuclein intermediate because PQ has been shown to induce a conformational change in α-synuclein and significantly increase the rate of the formation of α-synuclein fibrils *in vitro* (Uversky *et al.*, 2001). *In vivo*, rodent studies have shown that the administration of PQ induced an increase in α-synuclein levels in the brain. These results suggest that the upregulation of α-synuclein as a result of toxic insult and the direct interactions between the protein and environmental agents are potential mechanisms leading to α-synuclein pathology in neurodegenerative disorders (Manning-Bog *et al.*, 2002).

4.2 PINK1/PARKIN (PARK6/PARK2) and paraquat

Another hallmark PD characteristic is mitochondrial dysfunction. In *post-mortem* analysis in the substantia nigra, some patients with PD showed complex I deficiency (Schapira *et al.*, 1989). In addition, the oxidative stress was higher in patients with parkinsonism (Jenner, 2003). In this sense, *PINK1* (*PARK6*) and *Parkin* (*PARK2)* are 2 genes related to PD that may be involved in the regulation of mitochondrial homeostasis.
Parkin mutations were first linked to an autosomal recessive juvenile-onset form of PD in Japanese families (Kitada *et al.*, 1998; Matsumine *et al.*, 1997). Numerous parkin mutations have been described, including deletions, multiplications and missense mutations (Hattori & Mizuno, 2004). Parkin protein acts as an E3 ubiquitin protein ligase in the UPS (Shimura *et al.*, 2000). Ubiquitination of proteins is essential to start to proteasomal protein degradation.

Therefore, parkin mutations should lead to an incorrect ubiquitination, blocking the degradation of the protein and leading to protein accumulation. Mutant parkin has been shown to impair mitochondrial function and morphology in human fibroblasts and to sensitise the cells to an insult with PQ, producing higher levels of oxidised proteins in the *Parkin*-mutant samples than in controls (Grunewald *et al.*, 2010). PQ has also been demonstrated to induce alterations in parkin solubility and result in its intracellular aggregation (C. Wang *et al.*, 2005).

PINK1 is a serine/threonine kinase capable of autophosphorylation. This protein has an N-terminal mitochondrial targeting signal (MTS), is synthesised as a full-length version (FL) and is processed into at least two cleaved forms (Δ1 and Δ2) (W. Lin & Kang, 2008). PINK1 is considered to be a mitochondrial protein with a role in protecting against oxidative stress and apoptosis in *in vitro* models (Valente *et al.*, 2004). Mutations in *PINK1* have been associated with autosomal recessive PD (Valente *et al.*, 2004) and with *PINK1* KO flies with motor deficits and disorganised mitochondrial morphology (Clark *et al.*, 2006). For the link between PINK1 and the toxicity of PQ, studies using silencer PINK1 have shown an increase in oxidative stress and ATP depletion and a higher sensitivity to PQ (Gegg *et al.*, 2009). Similar results have been observed in studies that examined PINK1 nonsense and missense mutations (Grunewald *et al.*, 2009).

4.3 DJ-1 (PARK7) and paraquat

DJ-1 is a small protein that belongs to the ThiJ/PfpI protein superfamily (S. Bandyopadhyay & Cookson, 2004) that was initially identified as an oncogene that interacted with H-Ras (Nagakubo *et al.*, 1997). The involvement of DJ-1 in neurodegeneration was found when it was discovered that the DJ-1 gene (*PARK7*) was the cause of autosomal recessive PD in a Dutch family (Bonifati *et al.*, 2003). Different pathogenic mutations have been identified in the *PARK7* gene, including truncation, exonic deletions and homozygous and heterozygous missense mutations (Hague *et al.*, 2003). L166P is the most dramatic point mutation, whereas other mutations, such as A104T and M26I, have a weaker destabilising effect on the protein structure. The L166P mutation is located in the centre of α-helix 7, which is a major part of the hydrophobic patch. This mutation has been shown to destabilise the dimeric structure of DJ-1 by promoting the unfolding of its C-terminal region, resulting in rapid degradation (Miller *et al.*, 2003; Moore *et al.*, 2003). However, the frequency of DJ-1 mutations was low, with it being estimated at approximately 1-2 % in early onset PD. The physiological function of DJ-1 is unclear, but it may have a role in protecting against mitochondrial damage in response to oxidative stress (Canet-Aviles *et al.*, 2004).

The link between DJ-1 and PQ exposure has been correlated with autophagy and the apoptotic process. An active role for DJ-1 in the autophagic response produced by PQ has been suggested. In a study using transfected cells exposed to PQ and DJ-1-specific siRNA, an inhibition of the autophagic events induced by the herbicide, the increased sensitisation during PQ-induced apoptotic cell death and the exacerbation of apoptosis in the presence of the autophagy inhibitor 3-methyladenine (R. A. Gonzalez-Polo *et al.*, 2009) had been shown. Interestingly, PQ-induced toxicity and proteasome dysfunction was potentiated in a DJ-1 deficiency (Lavara-Culebras & Paricio, 2007; Menzies *et al.*, 2005). In another study using DJ-1 null cells from the DJ-1(-/-) mouse embryos, DJ-1 null cells showed a resistance to PQ-induced apoptosis, including reduced poly (ADP-ribose) polymerase and procaspase-3. Therefore, DJ-1 could be important to maintain mitochondrial complex I, and complex I could be a key target in the interaction of PQ toxicity and DJ-1 in PD (Kwon *et al.*, 2011). In

DJ-1-deficient mice treated with PQ, decreased proteasome activities and increased ubiquitinated protein levels were found, and these pathologies were not observed in brain regions of normal mice treated with PQ (Yang *et al.*, 2007). In another mouse study, the loss of DJ-1 increased the sensitivity to oxidative insults but did not produce neurodegeneration. Similar results have been found when analysing *Drosophila melanogaster* mutants for the DJ-1 orthologous genes, DJ-1alpha and DJ-1beta, that resulted in increased sensitivity to PQ insults, reduced lifespan and motor impairments. However, these mutations did not lead to dopaminergic neuronal loss (Lavara-Culebras & Paricio, 2007)

4.4 LRRK2 (PARK8) and paraquat

In 2002, *PARK8* gene mutations were discovered as a major genetic cause associated with hereditary parkinsonism (Paisan-Ruiz *et al.*, 2004). The *PARK8* gene was associated with PD in studies of a Japanese Sagamihara family who responded positively to treatment with L-DOPA, which had parkinsonism that presented with an unknown aetiology of the disease (Funayama *et al.*, 2002). Other studies examined two additional families (German and Canadian) who also had an autosomal dominant, late-onset parkinsonism (Zimprich *et al.*, 2004).

In the LRRK2 structure, two functional domains, kinase and GTPase domains, were shown to be present. The G2019S mutation was present in the kinase domain specific to the binding site for Mg^{2+} (Kachergus *et al.*, 2005). This mutation facilitates the access of the kinase domain to its substrates, which increases autophosphorylation 2.5-fold the phosphorylation of other substrates, such as myelin basic protein (MBP), 3-fold for the LRRK2 autophosphorylation without the presence of this mutation (Jaleel *et al.*, 2007; West *et al.*, 2005), which is responsible for the increased toxicity of this molecule (Greggio *et al.*, 2006). In the GTPase domain, the R1441C has been the most studied mutation, and there is controversy as to the influence of GTPase mutations on the kinase activity that was observed in some studies in which the increase was similar (Guo *et al.*, 2007) or had no change (Jaleel *et al.*, 2007).

LRRK2 has been shown to play different roles in the cell; however, little information is available. Based on the data we found from the protein interactions, there was a relationship between LRRK2 and cytoskeletal reorganisation (Gandhi *et al.*, 2008), maintenance functions and cell morphology (Plowey *et al.*, 2008), protein transport through synaptic vesicles (Shin *et al.*, 2008), and the ubiquitination process (Ko *et al.*, 2009). There have also been studies that relate LRRK2 and apoptosis (Ho *et al.*, 2009). Previous studies have shown a relationship between LRRK2 and other PD-related proteins, such as parkin (Ng *et al.*, 2009; W. W. Smith *et al.*, 2005), PINK-1 and DJ-1 (Venderova *et al.*, 2009) or α-synuclein (X. Lin *et al.*, 2009). The interaction of LRRK2 with PQ is not clear. Studies in *Drosophila melanogaster* in which the deletion of kinase domain of LRRK2 did not induce a higher sensitivity to the PQ stimulus has been shown (D. Wang *et al.*, 2008). In contrast, in *Caenorhabditis elegans* studies, the expression of human LRRK2 protein protected against PQ, which increased nematode survival in response to agents that cause mitochondrial dysfunction. However, protection by G2019S, R1441C, or kinase-dead LRRK2 was less effective than wild-type LRRK2 (Saha *et al.*, 2009). In another study with *Caenorhabditits elegans, PINK1* mutant genes have been observed in a minor mitochondrial length and increased PQ sensitivity of the nematode. Moreover, the mutants also displayed defects in axonal outgrowth of a pair of canal-associated neurons. We demonstrated that in the absence of lrk-1 (the *C. elegans* homologue

of human LRRK2), all phenotypic aspects of *PINK1* loss-of-function mutants were suppressed (Samann *et al.*, 2009)

5. Conclusion

PQ has been suggested as a potential aetiological factor for the development of PD. We have demonstrated that PQ was able to induce cell death by activating apoptotic machinery. However, PQ also displayed characteristics of autophagy, a degradative mechanism involved in the recycling and turnover of cytoplasmic constituents from eukaryotic cells. Finally, the cells suffered apoptotic death when the PQ remained. Whereas caspase inhibition retarded cell death, autophagy inhibition increased apoptotic cell death induced by PQ. These findings suggest a relationship between autophagy and apoptotic cell death following paraquat exposition and allows us to further investigate and increase our knowledge regarding the toxicity of paraquat and its relationship with the origin of PD.

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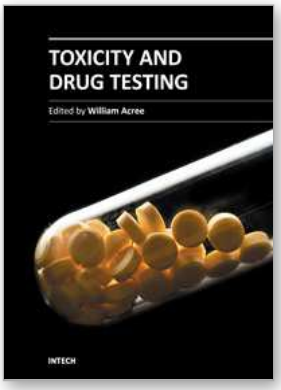

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Modern drug design and testing involves experimental in vivo and in vitro measurement of the drug candidate's ADMET (adsorption, distribution, metabolism, elimination and toxicity) properties in the early stages of drug discovery. Only a small percentage of the proposed drug candidates receive government approval and reach the market place. Unfavorable pharmacokinetic properties, poor bioavailability and efficacy, low solubility, adverse side effects and toxicity concerns account for many of the drug failures encountered in the pharmaceutical industry. Authors from several countries have contributed chapters detailing regulatory policies, pharmaceutical concerns and clinical practices in their respective countries with the expectation that the open exchange of scientific results and ideas presented in this book will lead to improved pharmaceutical products.

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