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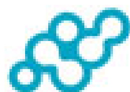

Health Sciences Research Centre

ANTIOXIDANT ENZYMES AND THEIR ROLE IN PARAQUAT INDUCED DOPAMINERGIC TOXICITY

- CONTRUBUTION OF NADPH OXIDASE 1

Ana Francisca da Silva Morais

Dissertation presented to University of Beira Interior, in partial fulfillment of the requirements for Master Degree in Biochemistry

Supervision the Professor Graça Baltazar
Co orientation: Ana Clara Cristóvão

Covilhã, 2010

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Departamento de Química

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"Carpe Diem"

Odes- Horácio

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All data present in this dissertation are my responsibility.

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LIST OF ABBREVIATIONS

AAV2: Adeno-associated virus serotype 2;

DA: dopamine;

DAergic: dopaminergic;

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase;

GPx: glutathione peroxidase;

GSR: glutathione reductase;

H_2O_2: hydrogen peroxide;

i.p.: intraperitoneal;

Lewy Bodies: LBs

MPP^+: 1-methyl-4-phenylpyridinium;

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine;

mRNA: messenger Ribonuclei acid

MTT: 3-(4,5-dimethylthiazal-2-yl)-2,5-diphenyl-tetrazolium bromide;

NADPH: Nicotinamide adenine dinucleotide phosphate;

Nox: NADPH oxidase;

$O2^{\bullet +}$: superoxide anion;

PQ: paraquat;

PD: Parkinson's disease;

ROS: Reactive oxygen species;

RT-PCR: Reverse Transcriptase Polymerase Chain Reaction;

shRNA: small hairpin Ribonucleic acid;

SEM: standard error of the mean;

SOD: superoxide dismutase;

SN: substantia nigra;

SNpc: substantia nigra pars compacta;

RESUMO

Doença de Parkinson (DP) é uma doença neurodegenerativa crónica progressiva, caracterizada pela degeneração de neurónios dopaminérgicos nigroestriatais na *substantia nigra pars compacta* (SNPC). Diversas condições ambientais, tais como a exposição a pesticidas, como Paraquato (PQ) são considerados factores de risco para DP. O tratamento de modelos animais com PQ induz alterações características do parkinsonismo. A toxicidade celular do PQ é, em parte, devido às reacções do ciclo redox que levam à geração de espécies reactivas de oxigénio (ROS), principalmente o peróxido de hidrogénio e o radical hidroxilo e consequente dano celular. Para lidar com as reacções oxidativas, enzimas antioxidantes como a superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase estão presentes nas células para equilibrar os mecanismos oxidativos. Portanto, a fim de neutralizar o stress oxidativo, indutores ROS podem também provocar um aumento dos níveis de enzimas antioxidantes. O PQ induz os seus efeitos tóxicos sobre as células N27 activando o sistema NADPH oxidase (Nox), particularmente Nox1, que, por sua vez, gera ROS. Levando em conta estes dados levanta-se a possibilidade que a Nox1 dopaminérgica possa estar envolvida na regulação da expressão de enzimas antioxidantes. Foram avaliados os níveis de mRNA de SOD e CAT nas células N27 após 1 e 6h de exposição ao PQ. Os níveis de proteína foram avaliadas após um período de incubação com PQ, de 16 e 24h, com ou sem a presença de apociana, um inibidor de Nox1. Estudamos também os níveis de proteína da SOD e CAT no SN de ratos expostos ao PQ. A estimulação das células N27 com baixas concentrações de PQ aumenta os níveis de expressão de mRNA da SOD, enquanto o aumento dos níveis de mRNA para a CAT foram observadas em células expostas a altas concentrações de PQ para 1h. No entanto, os níveis de proteína da CAT aumentaram na incubação por 24 horas, com 1000 mM PQ, e os níveis de SOD aumentaram com baixa concentração de PQ. O aumento dos níveis de ambas as proteínas, SOD e CAT, induzidas por PQ foram reduzidas *in vivo* pelo silenciamento Nox1, resultados concordantes com os dados da imunohistoquímica. Em conclusão, com o presente estudo, nós demonstramos que PQ aumenta a expressão SOD e CAT e que a Nox1 pode estar envolvida na regulação dessas enzimas antioxidantes.

Palavras-chaves: Doença de Parkinson, Paraquato, NADPH oxidase, Superoxido dismutase, Catalase.

Abstract

Parkinson´s Disease (PD) is a chronic progressive neurodegenerative disease; characterized by degeneration of nigrostriatal dopaminergic neurons in the *substantia nigra pars compacta* (SNpc). Several environmental conditions such as exposure to pesticides like Paraquat (PQ) are considered risk factors for PD. Treatment of animal models with PQ induces changes characteristic of parkinsonism. The cellular toxicity of PQ is, in part, due to its redox cycle of reactions that lead to the generation of reactive oxygen species (ROS), mainly hydrogen peroxide, and hydroxyl radical and to the consequent cellular damage. To cope with the many oxidative reactions, antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and gluthathione peroxidise are present in cells to balance the oxidative mechanisms Therefore, in order to counteract the oxidative stress, ROS inducers can also trigger an increase of antioxidant enzymes levels. PQ induces its toxic effects on N27 cells by activating the NADPH oxidase (Nox) system, particularly Nox1, which, in turn, generates ROS. Taking in account this data we raise the possibility that dopaminergic Nox1 may be involved in the regulating the expression of antioxidant enzymes. We assessed mRNA levels of SOD and CAT after 1 and 6h exposure to PQ in N27 cells. Protein levels were evaluated after an incubation with PQ during 16 and 24h, with or without the presence of apocynin, an inhibitor of Nox. We also studied SOD and CAT protein levels in the SN of rats exposed to PQ. Stimulation of N27 cells with low concentrations of PQ increased SOD mRNA expression levels, whereas increases of CAT mRNA levels were observed in cells exposed to high concentrations of PQ for 1h. . However, CAT protein levels were increased by incubation, for 24hr, with 1000 μM PQ, and SOD levels were increased by incubation with low concentration of PQ. The increase of both SOD and CAT protein levels induced by PQ was reduced by the in vivo Nox1 silencing, results concordant with the the immunohistochemistry data. In conclusion, with the present study, we demonstrated that PQ increases SOD and CAT expression and that Nox1 may be involved in the regulation of these antioxidant enzymes.

Keyworks: Parkinson´s Disease, Paraquat, NADPH oxidase, Superoxide dismutase, Catalase.

CHAPTER 1

INTRODUCTION

1.1 Parkinson's disease (PD)

PD is a chronic progressive neurodegenerative disease, first described by James Parkinson in 1817, affecting at least 1% of the population over the age of 55. It is the second most common neurodegenerative disorder after Alzheimer's disease, with 5–24 new cases per 100,000 population diagnosed every year (Rajput 1992). PD most commonly manifests between the fifth and seventh decade of life with resting tremor on one or both sides of the body, bradykinesia (slowness of movement), rigidity (stiffness of limbs), and abnormal postural reflexes (gait or balance problems) (Uversky 2004).

Currents treatments, such as administration of L-DOPA, are only symptomatic and do not stop or delay the progressive neuron loss (Bossy-Wetzel *et al.* 2004).

1.1.1 Neuropathology features of PD

The pathology of PD is not fully understood (Dawson and Dawson 2003; Dinis-Oliveira *et al.* 2006) but based on morphologic and anatomical findings, PD is characterized by massive degeneration of nigrostriatal dopaminergic (DAergic) neurons in the *substantia nigra pars compacta* (SNpc) (figure1.1) with the resultant deficiency in dopamine (DA) at the nerve terminals in the striatum (Ruberg *et al.* 1995; Schapira 2005). The common feature of PD is the degeneration of the neural connection between the *substantia nigra* (SN) and the striatum, two essential brain regions in maintaining normal motor function (Dinis-Oliveira *et al.* 2006). The striatum receives its DAergic input from neurons in SNpc via the nigrostriatal pathway (Moore *et al.* 1971). Progressive degeneration of the nigrostriatal DAergic pathway results in a profound deficiency in striatal DA (Dinis-Oliveira *et al.* 2006). Besides the loss of SN neurons, another important pathological feature of PD is the presence of neuronal cytoplasmatic inclusions known as Lewy bodies (LBs) in some surviving nigral DAergic neurons (Gibb and Lees 1988; Marsden 1994).

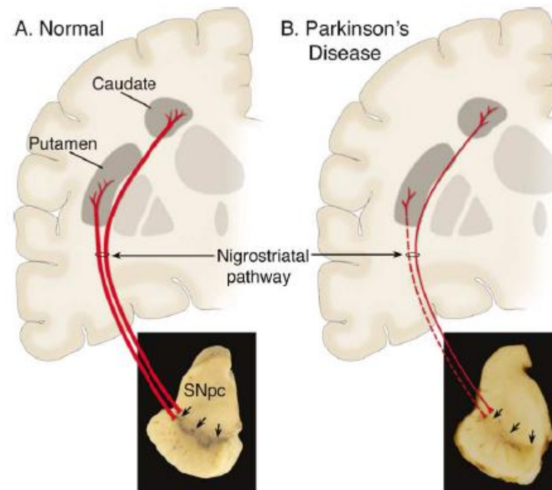

Figure 1.1- Neuropathology of PD.
Schematic representation of the normal (A) and the disease (B) nigrostriatal pathway. Photograph shows the depigmentation of the SNpc due to DAergic neurons degeneration (B).From Dauer (2003)

1.2 Etiology

While the specific etiology of PD remains unknown, ageing is the strongest risk factor for developing the disease. Genetic susceptibility and environmental risk factors also have come under critical investigation. It as been estimated that 5-10% of all parkinsonian cases are familial with increased frequency of the disease among relatives (Olanow and Tatton 1999). It is unclear whether the familial component of PD reflects a genetic background or exposure to the same environmental toxins, but most likely it is the combination of both (Semchuk *et al.* 1993).

In recent years, several genes that contribute to both autosomal dominant and recessive inheritance in familial PD were identified; including α-synuclein, parkin, DJ-1, LRRK2, and PINK1 (Abeliovich and Flint Beal 2006).

Other epidemiological studies have recognized several environmental conditions as risk factors for PD including rural living, farming, drinking well water, and exposure to agricultural chemicals (Firestone *et al.* 2005). Furthermore, a number of environmental toxicants including metals, solvents, and carbon monoxide have been associated with PD development (Uversky 2004). Nevertheless, pesticide exposure has received by far the biggest attention, presumably due to the implications of the widespread use of such agents on global public health. A comprehensive study of epidemiologic and toxicologic literature suggests a consistent correlation between pesticide exposure and PD with the strongest association resulting from long duration exposures (Brown *et al.* 2006). With respect to implications on human health risk, a

number of pesticides have come under intense scrutiny regarding their potential neurotoxic actions resulting in the development of the “environmental hypothesis of PD.” This hypothesis speculates that chemical agents present in the environment are capable of selectively damaging DAergic neurons, thus contributing to the development in PD. Recently; the use of pesticides in toxicant-based models of PD has become increasingly popular and provided valuable insight into the neurodegenerative process.

Environmental toxins, pesticides such as rotenone, maneb (MB) and Paraquat have been shown to induce PD-like symptoms in experimental animals (Somayajulu-Nitu *et al.* 2009).

1.3 Paraquat

Paraquat (1,10-dimethyl-4,40-bipyridilium dichloride; PQ) is a nonspecific commercial herbicide initially synthesized in 1961, and is one of the most widely applied herbicides in the world (Cory-Slechta *et al.* 2005). PQ is a charged molecule, with a hydrophilic structure. Shimizu and co-workers (2001) showed that PQ is taken up into the brain by the neutral amino acid transport system, and then transported into brain cells in a Na^+-dependent manner. Once in the brain, PQ is selectively taken up into terminals of DAergic melanin-containing neurons in the SN through the DA transporter (Miller *et al.* 2009).

The potential contribution of PQ to DAergic neurodegeneration was initially considered following the discovery that MPP^+ exposure resulted in the loss of DA neurons and in behavioral symptoms which were remarkably similar to those evidenced in PD. The structural similarity of the herbicide with the DAergic neurotoxin MPP^+ (figure 1.2) has been suggested as a potential etiologic factor in PD (Brooks *et al.* 1999; Cory-Slechta *et al.* 2005). In fact, a study by Kang *et al.* (1997) showed that PQ is more cytotoxic than MPP^+. Consequently, it has been suggested that human exposure to PQ may contribute to the loss of DA neurons and the etiology of PD and epidemiological studies support this association (Semchuk *et al.* 1991). Furthermore, PQ is able to elicit in addition to a dose-dependent decrease in SN DAergic neurons some decrease in striatal DA nerve terminal density and to induce neurobehavioral syndrome characterized by reduced ambulatory activity (Brooks *et al.* 1999).

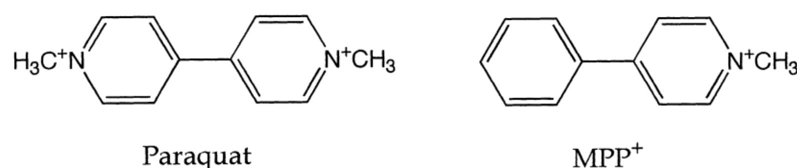

Figure 1.2 Chemical structures of PQ and MPP^+. From Dinis-Oliveira (2006)

The most frequent exposure to PQ occurs either accidentally or intentionally, in humans and animals by ingestion or through direct skin contact. Irrespective of its route of administration in mammalian systems, PQ is rapidly distributed in most tissues. The compound accumulates slowly via an energy-dependent process. Excretion of PQ, in its unchanged form, is biphasic, owing to lung accumulation and occurs largely in the urine and, to a limited extent, in the bile. Biotransformation of PQ is, in general, poor in all species studied and the excreted compound is unchanged (Suntres 2002).

1.3.1 PQ toxicity mechanism

PQ is an herbicide that inhibits mitochondrial complex I and III and perturbs the mitochondrial respiration chain causing impaired energy metabolism, proteasomal dysfunction and a widely used compound to induce intracellular ROS (Olesen *et al.* 2008; Miller *et al.* 2009).

The cellular toxicity of PQ is in part due to its redox cycle (figure 1.3). During redox cycling catalysed by cellular reductases, PQ undergoes a single electron reduction to form a PQ cation radical ($PQ^{\bullet+}$). $PQ^{\bullet+}$ is rapidly oxidized back to the parent compound with the concomitant transfer of the extra electron to molecular oxygen, forming superoxide anion ($O2^{\bullet+}$) (Bus and Gibson 1982).

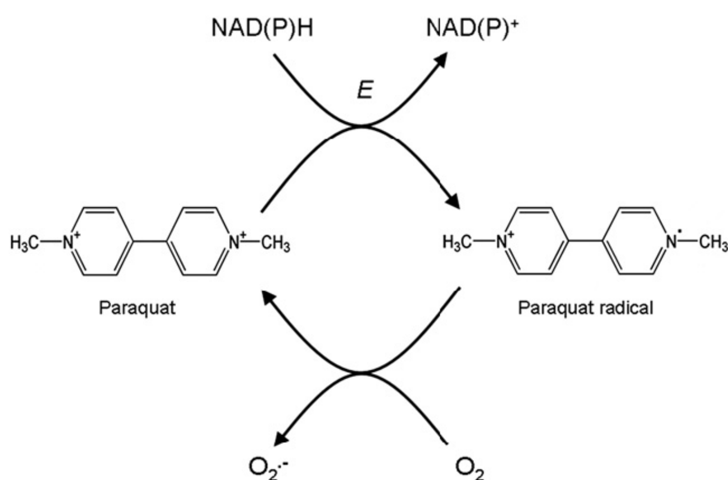

Figure 1.3 Schematic representation of the mechanism of PQ toxicity. PQ redox cycling: PQ undergoes a one electron reduction to the PQ radical via the oxidation promoted by different cellular reductases, E. The PQ radical is then immediately oxidized to the parent compound with the transfer of an electron to molecular oxygen, forming $O2^{\bullet+}$ (Black *et al.* 2008; Drechsel and Patel 2008).

This then sets off the well-known cascade of reactions leading to the generation of other reactive oxygen species (ROS), mainly hydrogen peroxide (H_2O_2) and hydroxyl radical and the consequent cellular deleterious effects (Youngman and Elstner 1981; Busch *et al.* 1998). Production of these ROS leads to intracellular oxidative stress and damage to cellular macromolecules including DNA, lipids and protein (Trouba *et al.* 2002). Functional groups on proteins are particularly susceptible to oxidation resulting in the formation of carbonyls which can lead to adduct generation with associated altered or loss of protein function (Dean *et al.* 1997; Levine 2002).

1.4 Oxidative stress

Neurochemically, PD is marked by mitochondrial complex I dysfunction and increased levels of oxidative stress (Dawson and Dawson 2003; Olanow 2007). In the past decades, several pathogenic mechanisms have been proposed for PD including oxidative and nitrosative stress, mitochondrial dysfunction, protein misfolding and aggregation, apoptosis, inflammatory responses and excitotoxicity (Swerdlow *et al.* 1996; Dawson and Dawson 2003). Oxidative stress has been implicated as a major mechanism of neuronal death in PD because DA-derived ROS and oxidized DA metabolites are toxic to nigral neurons. (Beal 2003; Wu *et al.* 2003) Brain constitutes only 2–3% of the total body mass, but it consumes 20% of body oxygen. Brain cells are particularly susceptible to oxidative damage due to high levels of polyunsaturated fatty acids in their membranes and the relatively low activity of endogenous antioxidant enzymes (Mariani *et al.* 2005). Aging is associated with increased oxidative stress and accumulation of oxidatively damaged biomolecules which gradually weakens cognition (Cardoso *et al.* 2005; Mariani *et al.* 2005). Many cellular reactions use molecular oxygen for catalysis and energy production. These reactions in turn produce ROS such as O_2^-, H_2O_2, hydroxyl radicals and peroxy radicals. To cope with the many oxidative reactions, antioxidants such as glutathione and vitamin E as well as antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) are present in cells to balance the oxidative mechanisms (figure 1.4). While these reactive species are important for execution of physiological functions, excessive production of ROS or insufficient antioxidant defense, particularly in the elderly, can damage cellular proteins, lipids and DNA and activate apoptotic pathways (Miller *et al.* 2009).

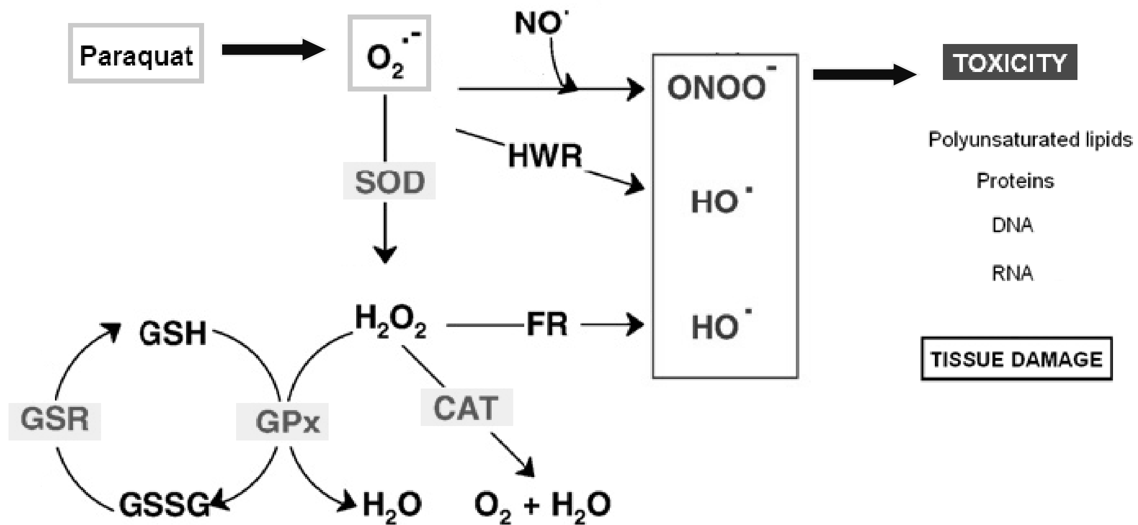

Figure 1.4. Detoxification of ROS by enzymatic antioxidants: Superoxide anion are metabolized to H_2O_2 by SOD. H_2O_2 is detoxified by CAT and/or various peroxidases, GPx. FR, Fenton reaction; HWR, Haber-Weiss reaction. Adapted from (Dinis-Oliveira *et al.* 2007)

1.5 NADPH oxidase

NADPH oxidase (Nox) enzymes are responsible to metabolize molecular oxygen and generate superoxide as a product (Wu *et al.* 2003). There is evidence that Nox is up-regulated in the SN in PD patients. Nox is comprised of three cytosolic components (p47phox, p67phox, and p40phox) and two membranes bound subunits, gp91phox and p22phox. Besides translocation of the cytosolic subunits to the membrane subunits, the enzyme complex also requires a small GTPase Rac for full function (Sumimoto *et al.* 2004). In recent studies, activation of Nox is considered a major source of superoxide in a number of neurodegenerative diseases including PD. Nox appears to be ubiquitously expressed in all brain regions and cell types including neurons and glial cells (Miller *et al.* 2009). The physiopathological importance of Nox has led many researchers to use several inhibitors of these enzymes, mainly apocynin in their experimental studies (Suntres 2002).

Recent studies demonstrate that PQ may induce its toxic effects on N27 DA cells by activating the Nox system, particularly Nox1, which, in turn, generates ROS and eventually results in DA neuronal death (Cristovao *et al.* 2009). Taking in account this data we raise the possibility that DAergic Nox1 may be involved in the regulation of antioxidant enzymes.

1.6 Antioxidants enzymes

Results of epidemiological studies demonstrated a relationship of certain human diseases, such as PD, with pesticide exposure and with changes in antioxidant enzymes (Lukaszewicz-Hussain 2008). Cellular antioxidants play a key role in the removal or detoxification, of ROS, which is essential for preventing oxidative damage. This system includes enzymes that act directly to detoxify ROS such as SOD, CAT and GPx, along with GSR (Black *et al.* 2008). These enzymes are cooperative in several aspects. At the most obvious level, SOD converts O_2^- into H_2O_2 and the latter must then be disposed of by CAT and peroxidase (Rao *et al.* 2000).

1.6.1 Superoxide Dismutase

The ubiquitous SOD catalyze the metabolism of superoxide to molecular oxygen and peroxide and thus are critical for protecting cells against the toxic products of aerobic respiration (Perry *et al.* 2010). Under normal circumstances, formation of superoxide anion produced by PQ or other chemicals is kept under control by the SOD enzymes. These include: the copper-zinc SOD, which is the primary species in the cytoplasm; the manganese SOD, which is the primary species in the mitochondria; and the extracellular SOD, which is the major form of SOD in extracellular fluids (Suntres 2002). The use of SOD as a treatment to ameliorate PQ-induced injuries has produced variable results. Exogenously-administered SOD conferred protection in young rats that had been challenged with PQ. Also, in adult rats, SOD reduced the mortality induced by PQ challenge from ≈ 80 to 45% over a 28-day period (Wasserman and Block, 1978). The protective effect of SOD against PQ toxicity has been attributed to its ability to scavenge the superoxide anion, generated from the redox cycling of PQ (Suntres 2002).

1.6.2 Catalase

CAT is major antioxidant enzyme responsible catalyzes the decomposition of H_2O_2 to water and oxygen (Lukaszewicz-Hussain 2008). H_2O_2 is detoxified by CAT and/or various peroxidases including GPx. CAT and GPx activities were determined in various regions of parkinsonian brains and control brains from patients with nonneurological diseases. The highest GPx activity was localized in the SN of the normal brain. In PD, the peroxidase activity was decreased in the SN, caudate and putamen. CAT activity was also reduced in the SN and putamen of the parkinsonian

brain. These enzyme changes may be causally related to the degeneration and depigmentation of the SN neurons in PQ exposed animals. (Ambani *et al.* 1975)

1.6.3 Glutathione peroxidase

GPx is an enzyme of major importance in the detoxification of peroxides in brain, protecting against oxidative stress. It helps preventing lipid peroxidation of cellular membranes by removing free peroxide from the cell. In the PD patients GPx activity was slightly but significantly reduced in several brain areas including SN. Although the magnitude of the GPx deficiency in PD SN was small (19% reduction), coupled with the reported marked deficiency of reduced glutathione it may represent one of the contributing factors leading to nigral DA neuron loss (Kish *et al.* 1985). GPx is shown as a 21-kD protein under reducing conditions in all tissues examined but is not in highly abundant in the human brain. Power and Blumbergs (2009) showed that unstructured LBs were enveloped with a layer of GPx-1 which partially colocalized with α-synuclein whereas concentric LBs had discrete deposits of GPx-1 around the periphery suggesting that GPx-1 may be involved in the degradation the LBs.

1.6.4 Glutathione Reductase

GSR is an enzyme that catalyzes the reduction of oxidized glutathione (GSSG) to glutathione (GSH). GSR is essential for the glutathione redox cycle that maintains adequate levels of reduced cellular GSH. GSH serves as an antioxidant, reacting with free radicals and organic peroxides, in amino acid transport, and as a substrate for the glutathione peroxidases and glutathione Stransferases in the detoxification of organic peroxides and metabolism of xenobiotics, respectively.

This homodimeric enzyme is a member of the flavoprotein disulfide oxidoreductases family. Each subunit has four domains; beginning at the N-terminus: an FAD-binding domain, an NADPH-binding domain, a central domain, and an interface domain. The active site of GSR is at the dimeric interface. Since the GSSG binding site is composed by residues from both subunits, only the dimeric form is active (Bashir *et al.* 1995).

1.7 Objective

Exposure to some pesticides, including PQ, increases the probability of developing PD. One of the mechanisms by which this compound exerts its toxicity involves the Nox system, particularly Nox1, which, in turn, generates ROS and eventually results in DA neuronal death. The aim of this study was investigate the effect of PQ on the expression of antioxidants enzymes and to clarify the role of Nox 1 on the regulation of these enzymes in the nigrostriatal DA pathway. For that we propose to evaluate the effect PQ in mRNA and protein expression levels of the enzymes, SOD, CAT *in vitro* using a DAergic cell line (N27 cells) as well as in a PQ rat model of PD. To determine the role of Nox 1 in controlling the expression of antioxidant enzymes induced by PQ, we will use, *in vitro* the Nox inhibitor, apocynin, and *in vivo*, the specific Nox1 knockdown in the rat SN by a adeno-associated virus system.

CHAPTER 2

METHODOLOGIES

2.1 Cells cultures

Immortalized rat mesencephalic dopaminergic cell (N27 cells) culture

The N27 cells were grown in RPMI 1640 medium containing 10% FBS, 100 units penicillin, and 50 μg/ml streptomycin, in a humidified atmosphere of 5 % CO_2 at 37 °C. N27 cultures were prepared for experiments by plating the cells on polystyrene tissue culture dishes at a density of $1x10^4$ cells/well in 96 well culture plates, 1×10^5 cells/well in 12 well culture plates, 1.5×10^5 cells/well in 6 well culture plates or 5×10^5 cells/100 mm dishes. When cells showed approximately 70% confluence, they were treated with different concentrations of PQ and/or apocynin (Sigma-Aldrich) for the indicated duration.

2.2 Cell treatments

Paraquat treatment

When N27 cells cultures reached approximately 70% confluency, they were treated for 1, 3, 6, 12, 16 or 24 h, with different concentrations of PQ (100, 500, 800, 1000, 1500μM, Sigma) and/or apocynin (5 μM).

2.3 Animals and treatment paradigm

In Vivo experiments

Male Wistar rats (Charles River (Wilmington, MA; USA); 8-10 weeks) were maintained in a temperature/humidity-controlled environment under a 12 hr light/dark cycle with free access to food and water. Each animal received four i.p. injections, separated by 1 day, of either vehicle (saline) or PQ (10 mg/kg of body weight), and according to previously published data (Manning-Bog et al., 2002; Harraz et al., 2008; Cristovao et al., 2009). In the studies aiming at selectively targeting Nox1 by adeno-associated virus (AAV)-mediated Nox1 knockdown in the rat SN, four weeks before starting PQ i.p. injection, animals were stereotaxically injected at the right SN using the following brain coordinates: mediolateral (ML), +2.0; anteroposterior (AP), -5.3; dorsoventral (DV), -6.8. Animals were organized into four groups:

1) Vector + vehicle: stereotaxically injected with AAV particles containing an empty vector (vector) and then i.p. injected with saline (Vehicle);

2) Vector + PQ: stereotaxically injected with the vector and then i.p. injected with PQ;

3) shNox1 + PQ: stereotaxically injected with AAV particles harboring Nox1 shRNA and then i.p. injected with PQ;

4) shNox1 + vehicle: stereotaxically injected with Nox1 shRNA and then i.p. injected with vehicle.

2.4 Western Blot analysis

CAT and SOD protein levels were determined by Western blot of samples obtained from tissue or cell homogenization using RIPA buffer (50 mM Tris/HCl, PH 8.0, 150 mM NaCl, 2 mM sodium orthovanadate, 1% Nonidet-P40, 0.5% sodium deoxycholate, 0.1% SDS, containing 1% of a protease inhibitor mixture (AEBSF, pepstatinA, E-64, bestatin, leupeptin, and aprotinin)). The concentration of protein was determined using the Bradford method and bovine serum albumin as standard (Biorad Protein Assay). Samples were denaturated by addition of sample buffer (100mm Tris, 100mM glycine, 4% SDS, 8M urea and 0,01% Bromophenol blue) and heated at 100ºC for 5min. 10μg of protein was separated by SDS-PAGE using a 10% resolving gel and electrotransferred onto polyvinyldiene diflouride (PVDF) membranes (Amersham Life Sciences) during 1hour 45min. After membrane blockade with 5% non fat milk (Regilait, France) in TBS-T (0,1% Tween 20 in a 20 mM Tris and 137 mM NaCl solution), for 60 min at room temperature, the membranes were incubated overnight at 4ºC with rabbit anti-CAT (1:1000; Abcam), rabbit anti-SOD (1:5000; Novus Biologicals) or mouse anti-tubulin (1:2000,Sigma) primary antibodies diluted in TBS-T. After being rinsed 4 times with TBS-T, membranes were incubated for 1 hour, at room temperature, with an anti-rabbit or anti-mouse secondary antibody (1:20,000 or 1:10,000 respectively; Amersham Biosciences) TBS-T. Membranes were them incubated with the ECF substract (ECF Western Blotting Reagent Packs, Amersham) for 3 min. Protein bands were detected using the Molecular imager FX system (Bio-Rad) and quantified by densitometric analysis using the Quantity One software (Bio-Rad).

2.5 Immunocytochemistry

After exposure to PQ, N27 cells were fixed in 4% paraformaldehyde for 20 min and permeabilized with 0.1% Triton X-100 in phosphate buffered saline (PBS) for 10 min. Blocking was performed by incubation with 20% FBS in PBS containing 0.1% Tween-20 for 90 min at room temperature. Then, the cells were incubated, for 1 h, at room temperature with the following primary antibodies, according to the aim of the experiment, rabbit anti-CAT (1:300) or rabbit anti-SOD (1:1000). After washing, cells were incubated for 1 h with anti-rabbit secondary antibody conjugated to Alexa Fluor® 488 (1:1000, Invitrogen). The images were analyzed on a Zeiss inverted microscope under a 40x magnification.

2.6 Immunohistochemistry

Following perfusion with saline and 4 % paraformaldehyde in PBS, brains were removed, and forebrain and midbrain blocks were fixed by immersion in 4% paraformaldehyde and cryoprotected by immersion in 30% sucrose in PBS with 0.02% sodium azide. After 2-3 days of immersion in sucrose tissues were frozen and stored at -80ºC. Serial coronal sections (40 μm) of the SN were cut on a cryostat, collected in cryopreservative solution (30% glycerol, 30% ethylene glycol and 0.02 M phosphate buffer), and stored at -20 ºC. For immunolabelling studies sections were incubated with blocking solution (5 % FBS and 0.3 % Triton X-100 in PBS, pH 7.5) and then incubated overnight at room temperature with primary antibodies. Finally, sections were incubated with secondary antibodies, in blocking solution, at room temperature for 1 hr. The primary antibodies used were rabbit polyclonal anti-CAT antibody (1:200), rabbit polyclonal anti-SOD2 antibody (1:500). The secondary antibodies were biotinylated anti-rabbit IgG, (1:200). The staining procedure was performed as indicated by the manufacturer of the Vectastain ABC kit (Vector Laboratories) and the reaction product visualized using 3,3'-diaminobenzidine (DAB, Sigma) reagent in Tris buffer saline containing 0.02% H2O2. *S*ections were air dried, cleaned in xylene and then mounted with the appropriate mounting medium. Analysis was performed on a Zeiss inverted microscope.

2.7 Cell viability assays

MTT reduction assay

To assess cell viability, MTT reduction was measured. For the assay cells were incubated with 0.5 mg/ml of 3-(4,5-dimethylthiazal-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Sigma) for 1- 1,5 hours at 37 ºC. MTT is converted by viable cells to a water-insoluble precipitate that is dissolved in 0.04 M HCl in isopropanol, and colorimetrically quantified at 570 nm using a microplate reader (Biorad).

2.8 Total RNA extraction

Total RNA was extracted from N27 cells using Trizol reagent (Invitrogen). Then 200 µl of Chloroform per 1 ml of TRI used were added and the samples were centrifuged at 12,000 G for 15 min at 4 ºC (Mikro 200R, Hettich Zentrifugen). Centrifugation resulted in the separation of the mixture in three phases, 1) red organic phase (proteins), 2) Interphase (DNA) and 3) colourless upper aqueous phase (RNA). For RNA isolation the colourless phase was collected to a new tube, and to precipitate the RNA, 500 µl of isopropanol per 1 ml of TRI used were added to precipitate the RNA. Finally, centrifugation at 12,000 g for 10 min at 4 ºC RNA pelleted the RNA (Mikro 200R, Hettich Zentrifugen). The RNA pellet was washed by adding 1 ml of cold ethanol (75%) per 1 ml of TRI and centrifuged at 7,500 g for 5 min at 4 ºC Mikro 200R, Hettich Zentrifugen). The resultant pellet was briefly air dried for 5-10 min. The RNA was then re-suspended in 20 µl of DEPC water, incubated at 55-60 ºC for 10 min and quantified. RNA concentration was determined spectrophotometrically by measuring the absorbance of samples at 260nm (*A*260).

2.9 RT-PCR

Single stranded cDNAs were synthesized by incubating 1 µg of total RNA for 5min at 65 ºC, then 10min at 25ºC and1hour at 37ºC with 1 unit/µL of superscript II reverse transcriptase. Random primers were used as primers. The samples were then heated at 75 ºC for 15 min to terminate the reaction. The cDNA obtained from 1 µg total RNA was used as a template for PCR amplification. Oligonucleotide primers were designed based on Genebank entries (table 2.1).

Table 2.1 Primer sequences of CAT, SOD, GPX2, GPX4, GSR and GAPDH

Gene	**Primer sequence**		**Pcr Product length (bp)**
	Sense 5´→3´	**Antisense 5´→3´**	
Rat CAT	5´-ACA TGG TCT GGG ACT TCT GG-3´	5´-CAA GTT TTT GAT GCC CTG GT-3´	197 bp
Rat SOD 1	5´-CCA CTG CAG GAC CTC ATT TT-3´	5´- CAC CTT TGC CCA AGT CAT CT-3´	216 bp
Rat GPx 2	5´-TGC CCT ACC CTT ATG ACG AC-3´	5´-TCG ATG TTG ATG GTC TGG AA-3´	169 bp
Rat GPx 4	5´-CCG GCT ACA ATG TCA GGT TT-3´	5´-ACG CAG CCG TTC TTA TCA AT-3´	166 bp
Rat GSR	5´-ACC ACG AGG AAG ACG AAA TG-3´	5´-ACG AAG TGT GAC CAG CTC CT-3´	204 bp

PCR reactions solutions contained 2.5 μl of 10x PCR buffer, 0.5 μl of each dNTP, 1 pmol each of forward and reverse primers, and 0.2 units of Taq polymerase (Roche Applied Science) to a final volume of 25.5μl. For GAPDH, the first step for amplification was performed at 95ºC for 5min, the second step was performed in 28 cycles for 30 sec at 95ºC, 30 sec 58ºC and 30sec at 72ºC. After the last cycle, samples were incubated for 5 min at 72ºC. For CAT, SOD, Gpx2, GPX4, GSR (Stab Vida) was performed the according with table 2.2. The number of cycle and annealing temperature (AT) were optimized. PCR fragments were analyzed on 1% agarose gel containing ethidium bromide and their amounts were normalized against amplified GAPDH. Each primer set specifically recognized only the gene of interest as indicated by amplification of a single band of the expected size.

Table 2.2 The three steps perfomed for PCR of CAT, SOD, GPX2, GPX4, GSR

1º Step	**95ºC**	**5 sec**	
	95ºC	**30 sec**	
2ºStep	***AT***	**30 sec**	Number of cycles
	72ºC	**30 sec**	
3º Step	**72ºC**	**5 sec**	

2.10 Data analysis and statistics

The data analysis was performed with the program GraphPad Prism version 5.0 for Windows, GraphPad Software, San Diego California, USA. Data are expressed as percentages of values obtained in control conditions, and are presented as mean ± S.E.M. of at least three animals for in vivo experiments or at least three experiments, from independent cell cultures. Statistical analysis was performed using the one-way ANOVA followed by Dunnett's test or Bonferroni's Multiple Comparison Test. Values of $p < 0.05$ were considered significant.

CHAPTER 3

RESULTS

3.1 Optimization of the PCR reaction

A successful PCR reaction requires an efficient amplification of the product. For that we needed to optimize the annealing temperature and the number of cycles to be used in PCR reaction to amplify our gene of interest.

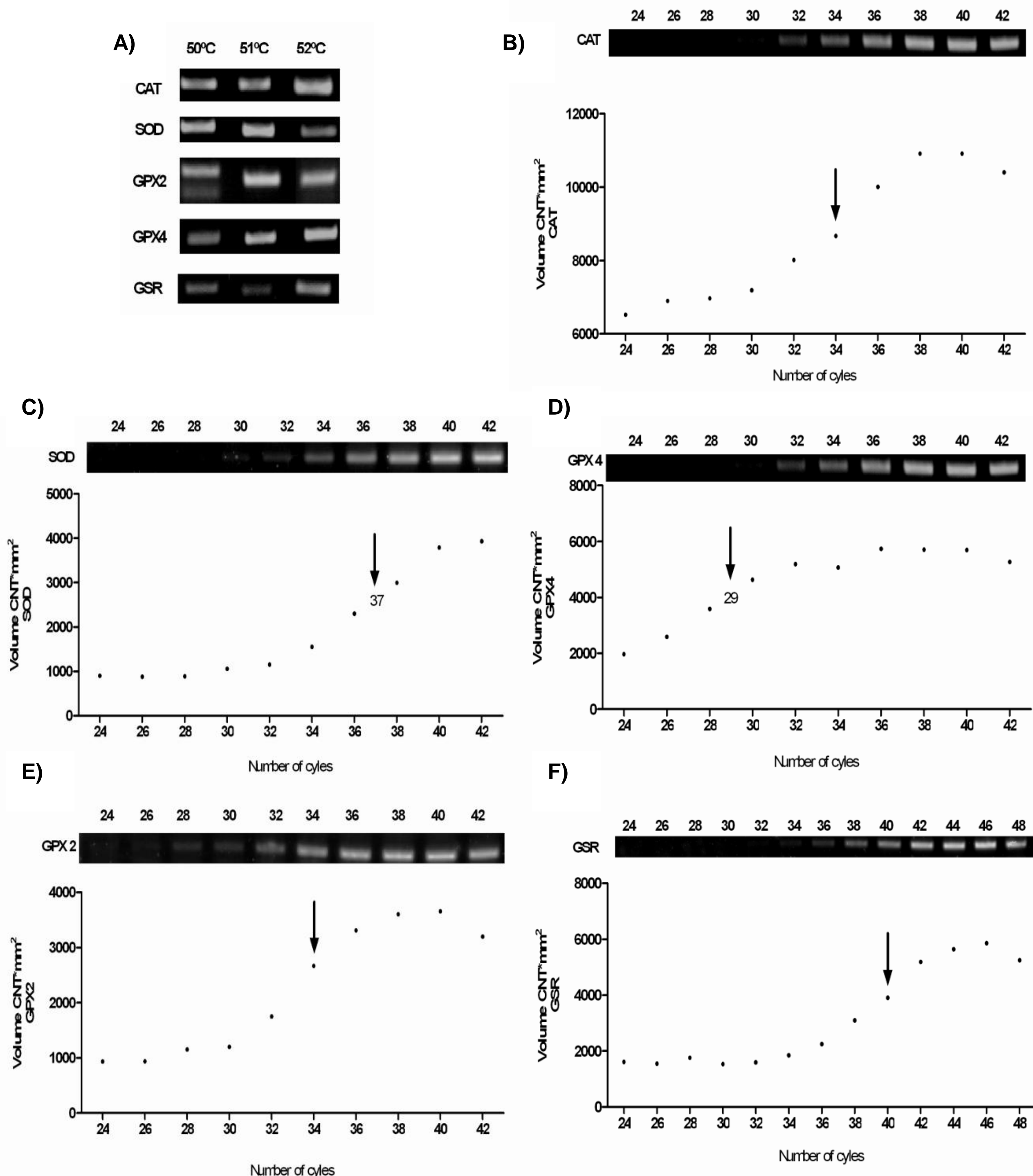

Figure 3.1 Optimization of the annealing temperature (A), and the number of cycles for SOD (B), CAT (C), GPX2 (E) , GPX4 (D), GSR (F), PCR reactions.

The temperature required for annealing primer depends on its base composition, length and concentration. The used primers contained 40-60% GC which ensures the stable binding of the primer to the template. We tested three annealing temperatures (50, 51 and 52ºC) for all primers and the temperature was chosen according to the intensity of the bands obtained (figure 3.1 A). The optimum number of cycles was obtained according with the PCR product yield (figure 3.1 B-F). Too many cycles can increase the amount and complexity of nonspecific background products (plateau effect) and too few cycles can produce low product yield. All the optimum conditions are summarized in Table 3.1.

Table 3.1. Summary of optimal conditions chosen for RT-PCR studies.

Gene	Temperature annealing (ºC)	Cycle number	Primer (µl)
Rat CAT	52	34	1
Rat SOD1	51	37	1
Rat GPX2	51	34	2
Rat GPX4	52	39	1
Rat GSR	52	40	1

3.2 SOD and CAT mRNA levels in N27 cells exposed to PQ

To investigate the effect of PQ in the mRNA expression of antioxidant enzymes, we evaluated mRNA levels of SOD and CAT in N27 cells treated with 100, 500, 800, 1000 or 1500 µM of PQ for 1 and 6 h.

3.2.1 Low concentrations of PQ induced increased in SOD mRNA expression levels expression in N27 cells.

After statistically analyzing the results, we found that mRNA expression of SOD was significantly increased in N27 cells exposed to concentrations of PQ below 800μM. As depicted in figure 3.2 A, after 1hour of incubation with PQ 100 or 500 μM, SOD mRNA increased to 128,3 ± 18,8% and 123 ± 3,5% of control, respectively. For longer incubation periods (6 hours), PQ exposure did not induced changes on SOD mRNA levels.

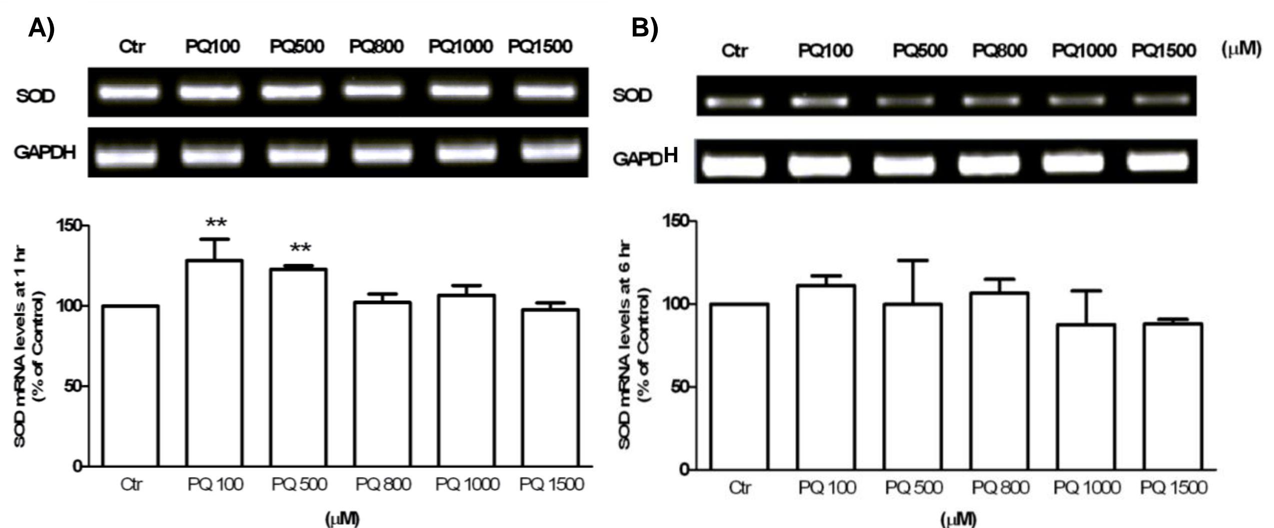

Figure 3.2 SOD mRNA levels detected by RT-PCR (upper panel), in N27 cells treated with PQ (100, 500, 800, 1000 or 1500μM) for 1hr (A) and 6h (B). Amplified PCR product were quantified with Quantity One software and then normalized to GAPDH, an internal control (lower panel). The results are expressed as percentage of control. Data shown represent the mean ± SEM of three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test. **P<0.01 vs. control cultures.

3.2.2 High concentrations of PQ induced increased CAT expression in N27 cells.

Similarly, with the results obtained for SOD, we observed that 1h, but nor 6h, exposure to PQ increased CAT mRNA levels. Cultures treated for 1h with 500 μM of PQ showed a 13.9 ± 9.3 % increase in CAT mRNA levels compared to control cultures (Figure 3.3 A, upper panel). Higher PQ doses, 800 μM and 1000 μM increased CAT mRNA levels by 17.8 ± 3 % and 25.8 ± 8.4 %, respectively (Figure 3.3 A, lower panel).

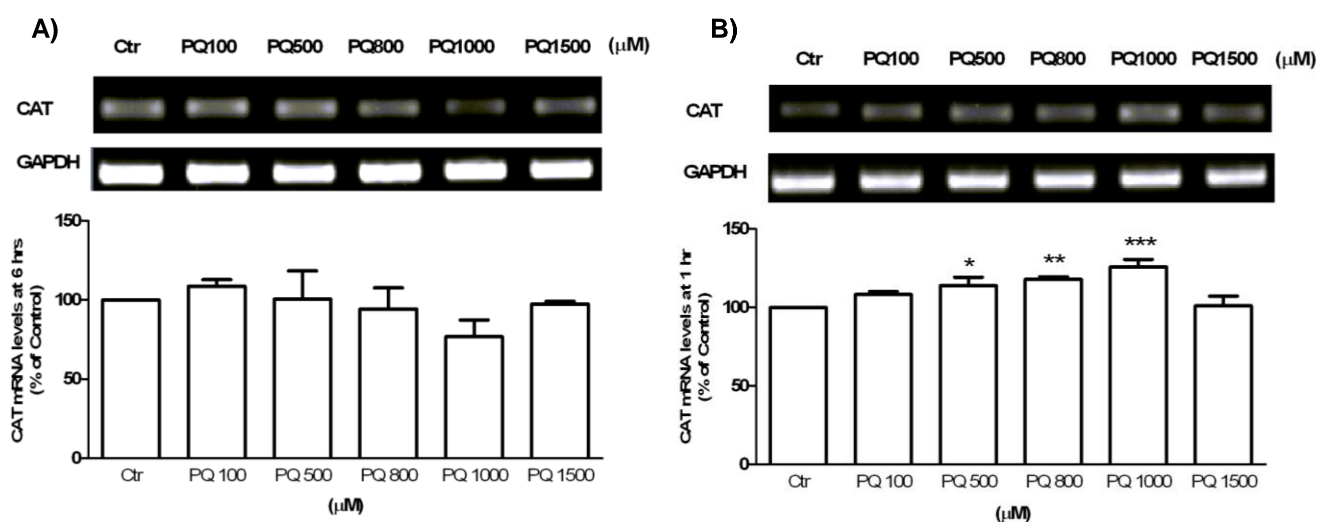

Figure 3.3 CAT mRNA levels detected by RT-PCR (upper panel), in N27 cells treated with PQ (100, 500, 800, 1000 or 1500μM) for 1hr (A) and 6h (B) and quantified with Quantity One software. The values were normalized using GAPDH as an internal control (lower panel). The results are expressed as percentage of control. Data shown represent the mean ± SEM of three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test. *P<0.05, **P<0.01, ***P<0.001 vs. control cultures.

3.4 Changes in SOD and CAT protein levels in N27 cells exposed to PQ

In order to clarify the possible role of NOX 1 on the effect induced by PQ on antioxidants enzymes, N27 cells were pre-treated for 1 h with 5 μM apocynin, a putative inhibitor of Nox1 activity followed by an incubation with different concentration of PQ. SOD and CAT protein levels were evaluated after 16 or 24h exposure to PQ. As depicted in figure 3.4 A and C, at 16h, no significant differences were found for SOD or CAT protein levels in any of PQ treated cultures. However, for longer incubation with PQ (24h) SOD levels were increased by 50.9 ± 11,5%, 42.1 ± 33.7% and 61.2 ± 24.5% at 100, 500 or 800 μM of PQ, respectively. CAT protein levels in cells incubated for 24hr, with 1000 μM of PQ were increased by 33.8 ± 23.65% relatively to the control (Figure 5.3 D). Even though in the presence of apocynin, SOD protein levels in cultures treated with different concentrations of PQ were reduced, these reductions were not significant (Figure 5.3B).

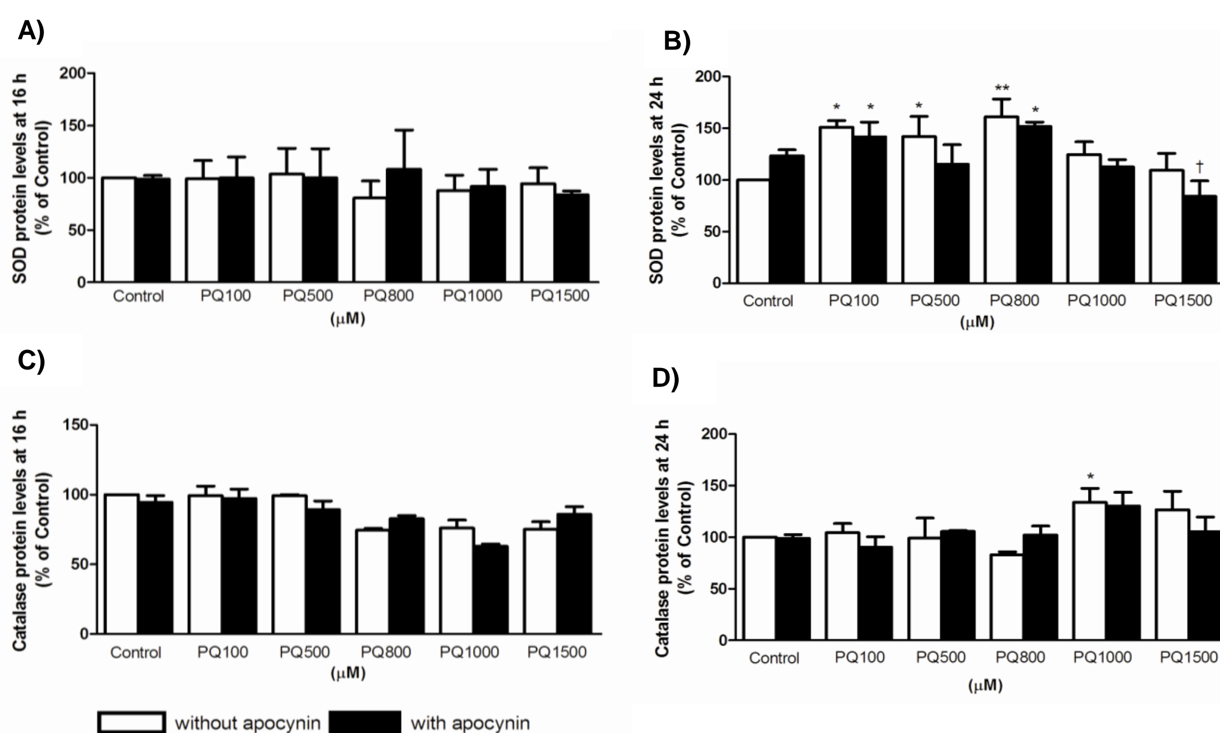

Figure 3.4 SOD and CAT protein levels assessed by western blot analysis in N27 cells treated with PQ (100, 500, 800, 1000 or 1500μM), in the presence or absence of apocynin. Protein levels were quantified using Quantity One software and then normalized using tubulin as an house keeping protein. Data are shown as the mean ± SEM of three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test. *P<0.05, **P<0.01, **P<0.001 vs. control cultures.

3.4 Effect of PQ on Cell viability

To evaluate the effects of PQ and apocynin on cell death, we investigated the levels reduced MTT in N27 cells for conditions in study. Cell death levels were assessed by measuring MTT reduction in N27 cultures pre-treated with 5 μM of apocynin for 1 hr and then treated with 100-1500 μM of PQ for 1, 6, 16 and 24h. The expected dose dependent decrease of MTT reduction induced by PQ was observed only for the 24h incubation period. The effect of apocynin on PQ induced MTT reduction was observed only at 16h incubation with the higher dose of PQ (1500 μM; figure 3.5 A). At 24h incubations pre treatment with apocynin promoted a significant decrease in PQ induced loss of cell viability. Cultures treated with 500, 800, 1000 or 1500 μM of PQ during 24 h, showed a decrease of MTT reduction levels of 29.5 ± 3.4%, 37.8 ± 19.6%, 46.9 ± 23.5% and 72.1 ± 23% of MTT respectively, compared with control cultures (figure 3.5 D). These toxic effects of PQ for concentrations the 500 and 800 μM was reversed by the presence of apocynin to 83.11 ± 11.8% and 74.98 ± 15% respectively.

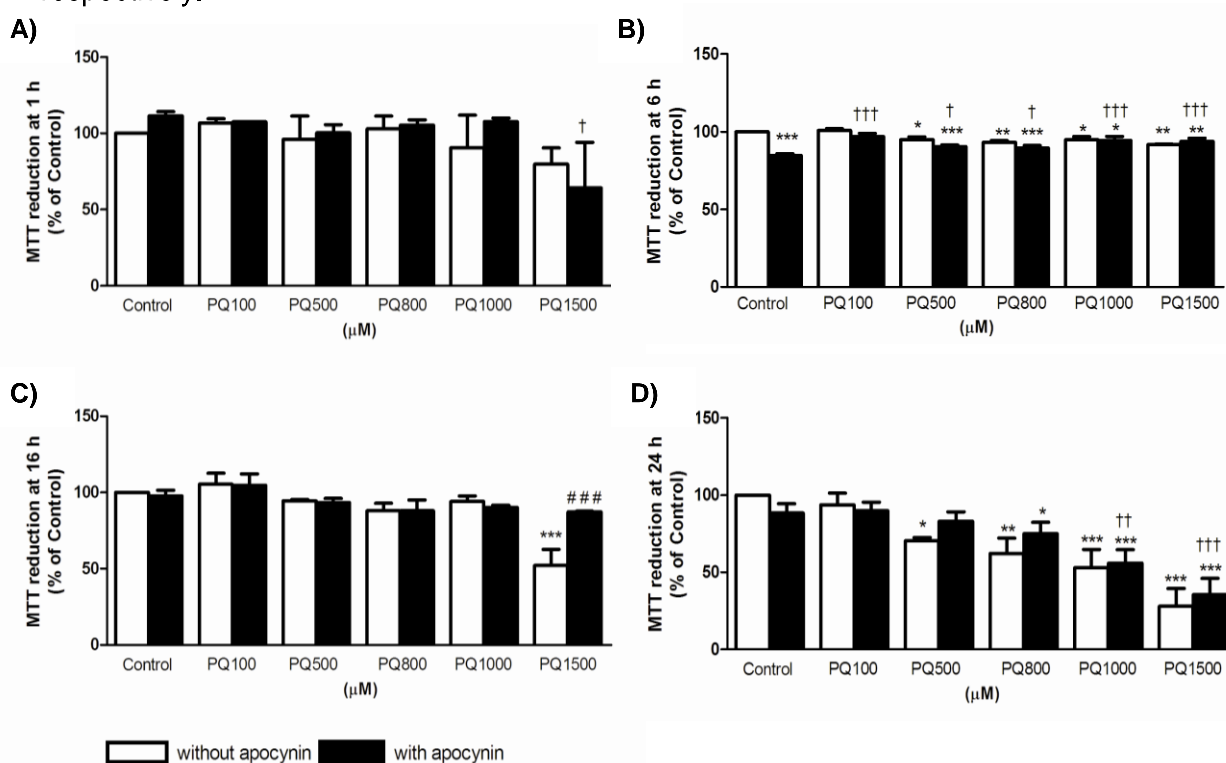

Figure 3.5. MTT reduction levels in N27 cells cultures pre-treated with 5 μM of apocynin for 1hr and then treated with PQ (100, 500, 800, 1000 or 1500μM). The results are expressed as percentage of their controls. Data are shown as the mean ± SEM of three independent experiments performed in triplicate. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test. *P<0.01, **P<0.001 and ***P<0.0001 vs. control cultures. †† P<0.01, and ††† P<0.001 vs. control pre-treated with apocynin only. # # #P<0.001 vs. cultures treated with the same concentration of PQ only.

3.5 Immunoreactivity for SOD and CAT after 24 hours exposure to PQ

The expression of SOD and CAT proteins by N27 cells in the presence of PQ was also analysed by immunocitochemistry. The results obtained showed an increase of SOD immunoreactivity in cultures exposed to 800 μM PQ and of CAT in cells exposed to 1000 μM PQ when compared with control cultures (figure 3.6 and 3.7).

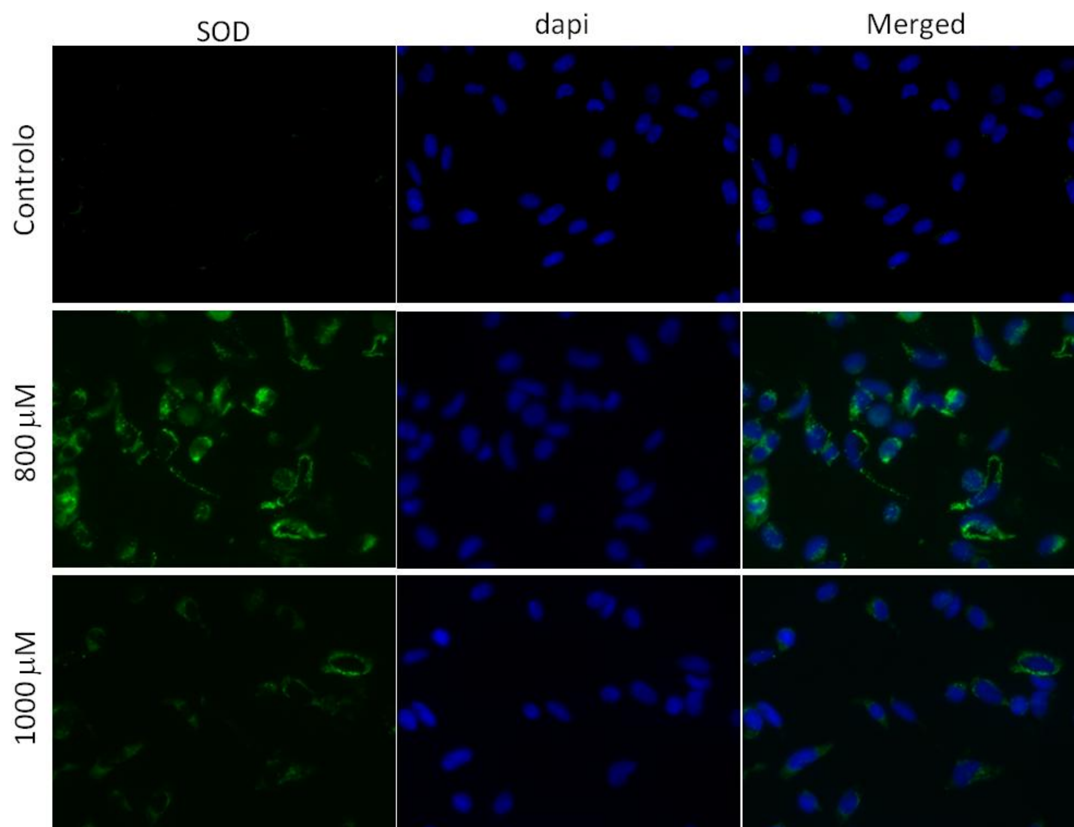

Figure 3.6 SOD immunoreactivity in N27 cells cutures exposed to 800 or 1000 μM of PQ for 24h. Magnification: 40x.

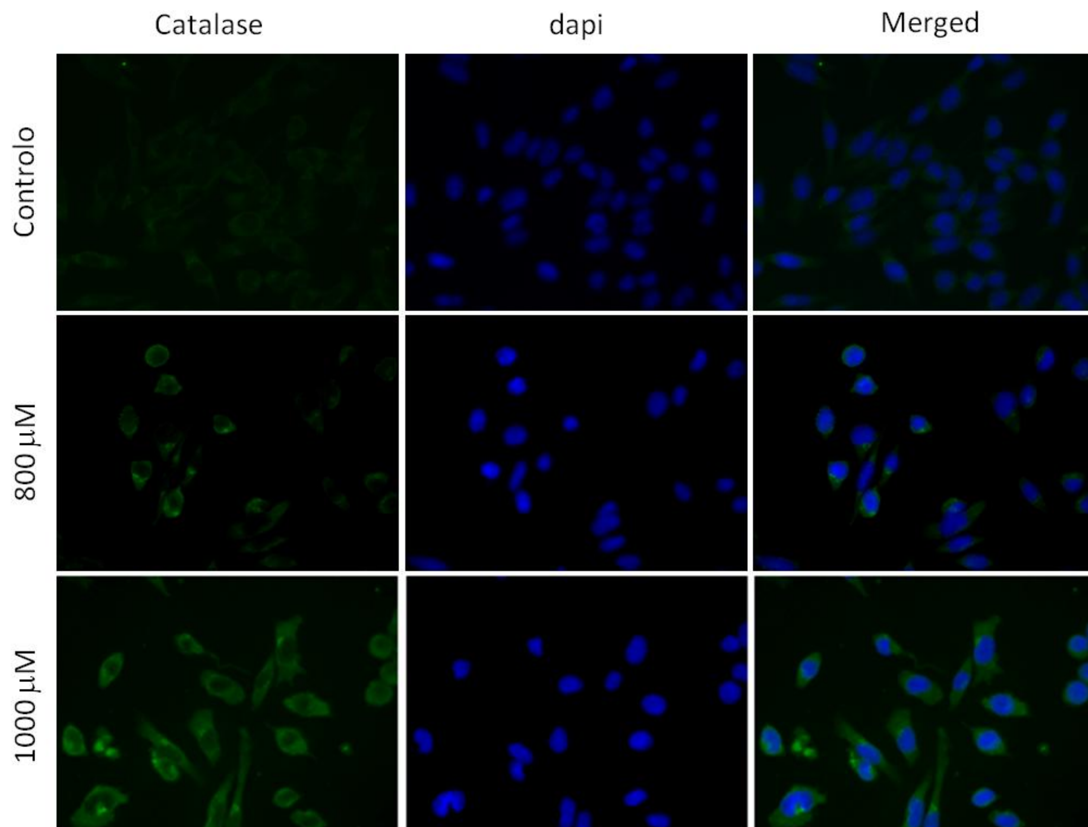

Figure 3.7 CAT immunoreactivity in N27 cells cutures exposed to 800 or 1000 μM of PQ for 24h. Magnification: 40x.

3.6 The effects of PQ on SOD and CAT protein levels in the rats SN.

A previous a study from our group has tested the PQ injection paradigm used in the present study and observed approximately 33% less DAergic cell in the SNpc of rats exposed to PQ compared with rats exposed to vehicle (Cristovao *et al.* 2009). In the current study, we have used the same injection paradigm to evaluate *in vivo* the effect of PQ in SOD and CAT protein levels in the SN of rats exposed to PQ and to evaluate the role of Nox1 in this effect. As depicted in figure 3.8 B and 3.9B, rats exposed to PQ shown 80.5 ± 28.3% and 52.8 ± 20.3% increase of SOD and CAT proteins levels, respectively, as determined by Western blot. These results were confirmed by immunohistochemistry data (figure 3.8 and 3.9) showing a decrease of SOD and CAT immunoreactivity in the SN of animals exposed to shNox1 + PQ, as compared to ones exposed to vector + PQ. Western blot data showed that Nox1 knockdown prior to PQ injection (shNox1 + PQ) reduced the increase of SOD and CAT levels promoted by PQ by 84.26 ± 6.6% and 66.42 ± 6% respectively (Figure 3.8 and 3.9 B). Taken together the results suggest that in situations of oxidative damage Nox1 may play a role in regulating SOD and CAT expression.

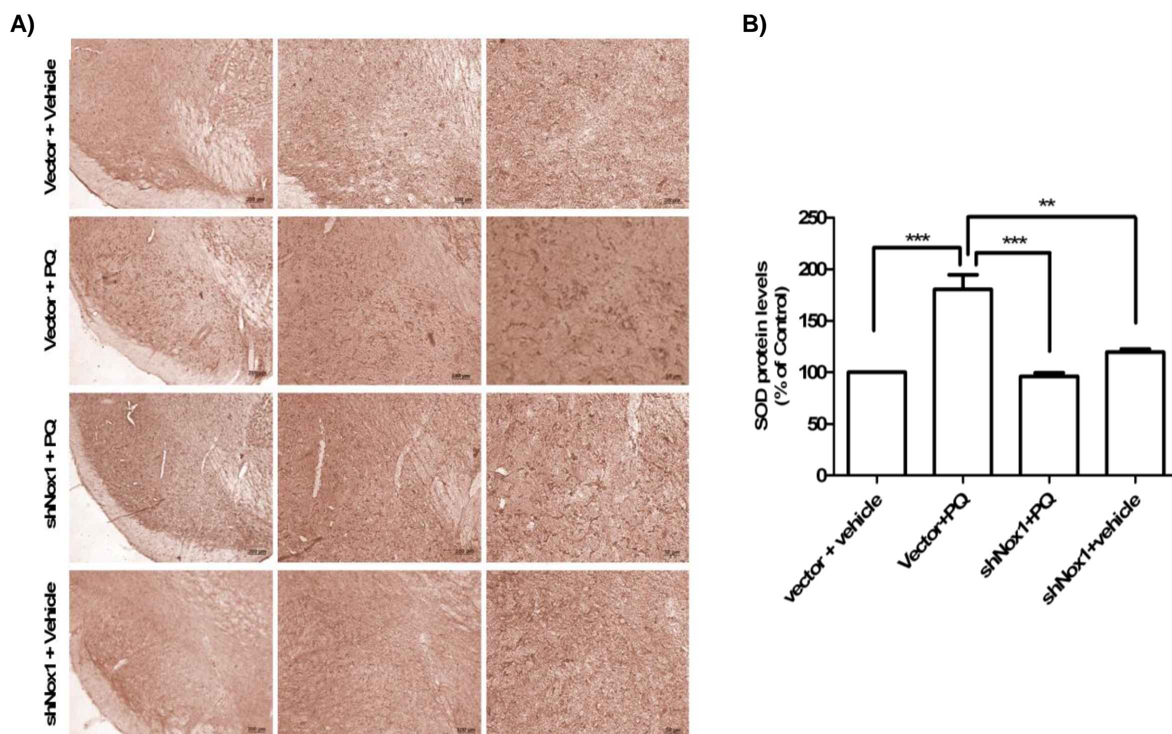

Figure 3.8 (A) Representative photomicrographs of SOD immunoreactivity in the ipsilateral SNpc of brain sections of the four experimental groups. Increased SOD immunostaining observed in vector + PQ group was significantly decreased by Nox1 knockdown as observed in shNox1 + PQ group. (**B**) Representative immunoblot and quantitative analysis of SOD protein

level. SOD protein was determined in total lysates of in the ipsilateral SN of rats by immunoblot analysis. SOD protein levels were quantified using Quantity One software and normalized against tubulin. The results are expressed as percentage of vector + vehicle. Data shown then mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test. **P<0.001 and ***P<0.0001

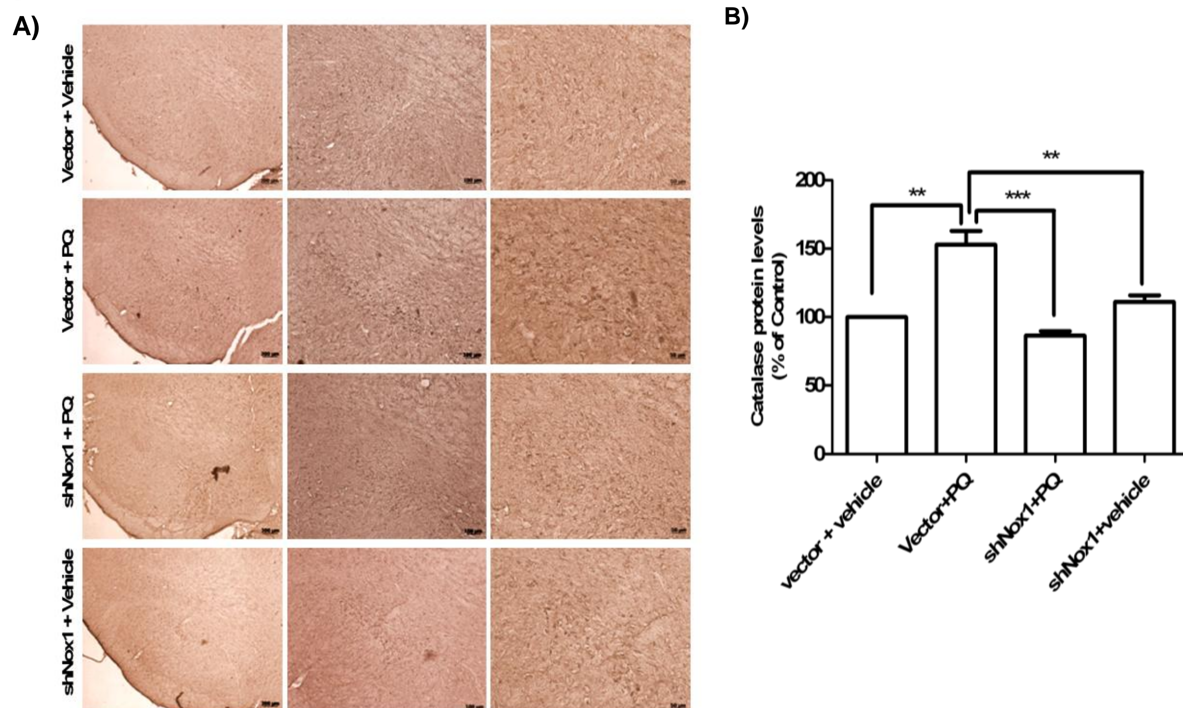

Figure 3.9 (A) Representative photomicrographs of CAT immunoreactivity in the ipsilateral SNpc of brain sections of the four experimental groups. Increased CAT immunostaining observed in vector + PQ group was significantly decreased by Nox1 knockdown as observed in shNox1 + PQ group. (**B**) Representative immunoblot and quantitative analysis of CAT protein level. SOD protein was determined in total lysates of in the ipsilateral SN tissues of rats, CAT protein levels were quantified using Quantity One software and normalized against tubulin. The results are expressed as percentage of vector + vehicle. Data shown then mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's Multiple Comparison Test. **P<0.01 and ***P<0.001

CHAPTER 4

DISCUSSION

Recent, investigations reported that PQ causes selective degeneration of DAergic neurons in the SNpc, reproducing a characteristic feature of PD (Fei *et al.* 2008). PQ promotes the increase of intracellular ROS, and therefore, in order to counteract the oxidative damage and to restore the cellular homeostasis, this can lead to activation or silencing of genes encoding regulatory transcription factors, antioxidant enzymes, and structural proteins. Therefore, we hypothesize that the antioxidant defences towards PQ detoxification will induce the transcription of several antioxidant enzymes. The first line of defence against ROS was enzymes antioxidants and we started study by SOD and Cat. The present study demonstrates an induction in antioxidant enzymes expression in cultures of the N27 cell line cultures exposed to PQ. The mRNA levels of SOD were up-regulated after 1 h of incubation with lower concentrations of PQ (100 or 500 µM) whereas mRNA levels of CAT did not suffer any changes. Since for higher concentration of PQ we were not able to measure significant increases in SOD mRNA expression we speculate that for those concentrations the amount of $O_2^{\bullet-}$ is too high leading to its quick metabolisation to H_2O_2, making unnecessary the over-expression of this enzyme but in stand favouring the expression of others, like CAT. In fact our results shown that for higher concentrations of PQ (800 and 1000 µM) the mRNA levels of CAT are significantly increased by PQ which did not occur at lower PQ concentrations.

It was previously reported that PQ-mediated increased of ROS levels and consequent N27 cells death were increased after an exposure of 24h to the pesticide, effects that were reversed in the presence of the putative NADH oxidase inhibitor, apocynin (Cristovao *et al.* 2009). In the present study the behaviour of SOD and CAT protein expression in the presence of PQ in N27 cells cultures were also investigated. SOD and CAT mRNA levels were increase after a short period of incubation with PQ (1h) whereas increased SOD protein levels were observed only after 24 of exposure to 100, 500 or 800 µM PQ. Increase CAT protein levels were observed only for the higher PQ concentrations, 800 and 1000µM also after an exposure of 24h. Immunocitochemistry data, confirmed an increase of SOD immunoreactivity in cultures exposed to 800 µM PQ and of CAT in cells exposed to 1000 µM PQ. The presence of apocynin did not caused a significant decrease of SOD or CAT proteins levels. Since we were not able to clearly evaluate the contribution of Nox1 probabily it could be due to the fact that we used a putative inhibitor of the Nox system, instead to perform the specific knochdown of Nox1, possible through the use of a siRNA strategy. The PQ-inducible ROS, can by itself be responsible for the transcriptional activation of SOD and CAT. However, the exact signal transduction pathway is still under investigation, as a large number of transcription factors, including NF-kB, AP-1, and the recently detected

Nrf2 have been shown to act through the antioxidant response elements (ARE) sequences (Schmidt *et al.* 1995; Lee *et al.* 2003). Subsequent studies have found that the Nrf2 may be the most potent transcription factor for the coordinate induction of phase II enzymes and antioxidant enzyme whose expression is under the influence of ARE (Lee *et al.* 2003). In this study, as demonstrated by the MTT test, 1500 μM of PQ was not lethal to N27 cells for up to 6h of incubation. Therefore, 100μM-1500 μM PQ allows generation of PQ-induced ROS related responses without inducing an immediate death of N27 cells. The results from the rat PQ modedel presented in this study showed that the results of SOD and CAT are coincident both by western blot and immunohistochemistry analysis. For both enzymes the increase in protein levels induced by PQ was reverted Nox1 downregulation. These results suggest that Nox1 may have a role in the regulation of SOD and CAT expression. It is possible that this regulation occurs through the Nrf2/Trx and/or ASK1/JNK pathways (which are involved in activation antioxidant response elements), since it was previously reported that these pathways are involved in the activation of cell death under oxidative conditions induced by PQ (Niso-Santano *et al.* 2010).

In conclusion, the results obtained in the present study demonstrated that PQ exposure increased SOD and CAT expression through a mechanism involving Nox1.

APPENDIX

1. Characterization of N27 cells

N27 cells are the first establishment of immortalized clones of DA-producing nerve cells in culture; they are from an immortalized line derived from the rat mesencephalon (Urban 2005). N27 cells express both tyrosine hydroxylase and the DA transporter, and produce measurable amounts of DA and may be useful not only for neural transplant but also for basic neurobiological studies (Prasad *et al.* 1994; Urban 2005).

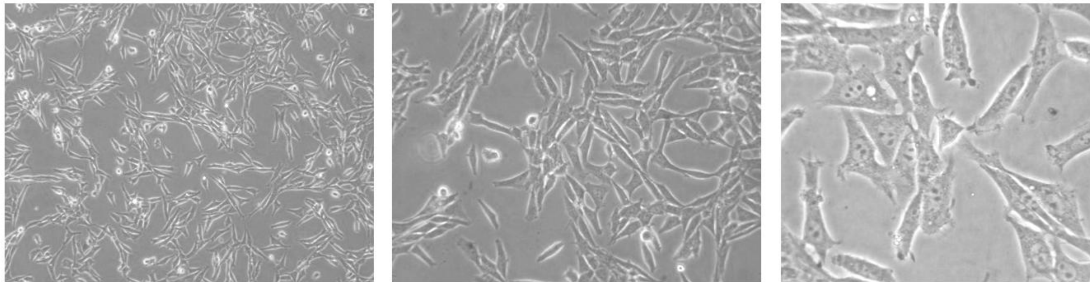

Figure 1.4- Photograph the of immortalized cell line, N27 cells, growing in RPMI medium. X100 (A) X200 (B) X400 (C)

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