NEW FORMULATION OF PARAQUAT WITH LYSINE ACETYLSALICYLATE

Safety improvement for mammalian and algae species with maintenance of the herbicidal activity

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Tese do 3º Ciclo de Estudos Conducente ao Grau de Doutor em Ciências Farmacêuticas – Especialidade: Toxicologia

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Aqueles que passam por nós, não vão sós, não nos deixam sós. Deixam um pouco de si, levam um pouco de nós

(Antoine de Saint-Exupéry)

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PUBLICATIONS

Papers in international peer reviewed journals

Theoretical background

I. **Baltazar MT**, Dinis-Oliveira RJ, Bastos ML, Duarte JA, Carvalho F (2011). "Antioxidant properties and associated mechanisms of salicylates." Curr Med Chem **18**: 3252–3264.

II. **Baltazar T**, Dinis-Oliveira RJ, Duarte JA, Bastos ML, Carvalho F, (2013). "Paraquat research: do recent advances in limiting its toxicity make its use safer?" Br J Pharmacol **168**, 44–45.

III. **Baltazar MT**, Dinis-Oliveira RJ, Bastos ML, Duarte, JA, Carvalho F. "Pesticides exposure as etiological factors of Parkinson´s Disease and other neurodegenerative diseases - a mechanistic approach." *Accepted for publication*.

Original Research

I. **Baltazar MT**, Dinis-Oliveira RJ, Guilhermino L, Bastos ML, Duarte JA, Carvalho F, (2012). New formulation of paraquat with lysine acetylsalicylate with low mammalian toxicity and effective herbicidal activity. Pest Manag Sci **69**:553–558.

II. **Baltazar MT**, Dinis-Oliveira, RJ, Martins A, Bastos ML, Duarte JA, Guilhermino L, Carvalho F, (2014). "The presence of lysine acetylsalicylate increases the safety of a paraquat formulation to freshwater primary producers: a case study with the microalga *Chlorella vulgaris*". Aquatic Toxicology 1**46**, 137–143

III. **Baltazar MT**, Dinis-Oliveira RJ, Bastos ML, Duarte JA, Carvalho F. "Lysine acetylsalicylate improves the safety of paraquat formulation in rats, by increasing its elimination, and preventing lung and kidney injury." *Submitted for publication*

Patent

Baltazar MT, Dinis-Oliveira RJ, Guilhermino L, Bastos ML, Duarte JA, Carvalho F. Formulação do herbicida paraquato. Portuguese Provisional Patent Nº 20121000028929.

ABSTRACT

Paraquat (1,1´-dimethyl-4,4´-bipyridylium dichloride; PQ) is an effective and widely used herbicide that has a proven safety record when appropriately applied to eliminate weeds. Over the last decades there have been numerous fatalities and for that reason PQ is considered one of the most toxic poisons involved in suicide attempts. The therapies currently used have failed to successfully ameliorate the burden regarding PQ poisoning. During the last years, our research group has been a reference to the scientific community in the field of PQ toxicity and development of antidotes. Sodium salicylate (NaSAL) and lysine acetylsalicylate (LAS) have been shown to have a multifactorial protection mechanism against PQ-induced toxicity, due to its ability to modulate inflammatory signaling systems, to prevent oxidative stress and to its capacity to complex with PQ. Although salicylates have conferred full survival when administered 2h after PQ intoxication in rats, in clinical practice sometimes patients might be admitted when lethal doses of PQ were already uptaken by pulmonary cells. An alternative approach to lowering PQ toxicity is to modify the PQ formulation. The formulation has faced some changes since its introduction such as the addition of a blue dye, a stenching agent, an emetic and an alginate. However, the existing formulations of PQ in the market are still highly toxic. The present thesis aimed to develop a safer PQ formulation with the incorporation of LAS as the antidote and preserving the herbicide effectiveness. To accomplish these aims the thesis is structured in three studies in order to evaluate i) the safety of the formulation to Wistar rats and maintenance of herbicidal activity; (ii) the safety of the formulation to the microalga *Chlorella vulgaris,* and (iii) the acute toxicity and mechanisms of toxicity, of the formulation in comparison with PQ.

For this purpose, the toxicity of the mixture of Gramoxone® (commercial formulation containing 20% PQ) and LAS in Wistar male rats and the herbicidal efficacy against grass lawn (50% of *Poa pratensis* and 50% of *Festuca arundinacea*) were evaluated. The survival rate of the PQ group was only 40%, while LAS provided effective protection, with full survival observed in the groups that received 125 mg/kg of PQ ion and 316 mg/kg of LAS. Moreover, the LD50 of the PQ+LAS formulation is higher than the LD50 of Gramoxone® (34.86 mg/kg *vs*. 187.5 mg/kg, respectively). Outstandingly, both formulations of PQ, either in the absence or in the presence of LAS, provided the same herbicidal activity against the tested herbal species.

In order to clarify the mechanisms involved in the protective effect of LAS, the formulation was administered to male Wistar rats by gavage, and sacrificed after 24 and 48h to evaluate the histological and biochemical biomarkers. LAS treatment caused a significant

reduction in PQ-induced lipid peroxidation, oxidative stress, and activation of nuclear factor kappa B (NF-κB) in lung. Also the PQ elimination was higher in the PQ+LAS group and was accompanied by the restoration of the normal values of urinary creatinine, proteins, and *N*-acetyl-β-glucosaminidase (NAG).

A considerable amount of the herbicides applied in crop fields enters into freshwater aquatic ecosystems leading to environmental contamination, potentially harming the aquatic organisms. Accordingly, it was also relevant to assess the safety of this new formulation to primary producers such as the microalga *Chlorella vulgaris*. PQ significantly inhibited *C. vulgaris* growth, an effect that was also significantly prevented by LAS at the proportion used in the formulation [1:2 (mol/mol)], while the highest protection was achieved at the proportion of 1:8.

In conclusion, the present thesis successfully achieved the goals proposed by developing a formulation of PQ that demonstrated to be: (i) safer to Wistar rats, (ii) equally effective against weeds and, (iii) less toxic to microalgae.

Keywords: paraquat, herbicide, antidote, lysine acetylsalicylate, algae, *Chlorella vulgaris*

RESUMO

O paraquato (dicloreto de 1,1´-dimetil-4,4´-bipiridilo, PQ) é um herbicida eficaz e amplamente utilizado que tem um histórico de segurança comprovado quando aplicado de forma adequada para eliminar as ervas daninhas. Ao longo das últimas décadas, o PQ tem causado inúmeras fatalidades e, por essa razão, é considerado um dos compostos mais tóxicos usados em tentativas de suicídio. As terapias usadas atualmente têm sido ineficazes na redução das fatalidades resultantes do envenenamento com PQ. Durante os últimos anos, o nosso grupo de investigação tem sido uma referência para a comunidade científica em relação ao estudo da toxicidade do PQ e desenvolvimento de antídotos para o tratamento das intoxicações. O salicilato de sódio (NaSAL) e o acetilsalicilato de lisina (LAS) possuem um mecanismo de proteção multifatorial contra a toxicidade induzida pelo PQ, devido à sua capacidade anti-inflamatória, capacidade antioxidante e à sua capacidade de complexar com o PQ. Embora os salicilatos sejam capazes de conferir uma sobrevivência de 100% quando administrados 2h após a intoxicação com PQ em ratos, na prática clínica quando os pacientes são admitidos, a concentração pulmonar atingida pode já ser letal. O desenvolvimento de formulações com menor toxicidade poderá constituir uma alternativa eficaz para as intoxicações voluntárias com PQ. Desde que foi introduzida no mercado, a formulação de PQ tem sofrido alterações ao longo dos anos, tais como, a adição de um corante azul, um agente modificador de odor, um emético e um alginato. No entanto, as formulações de PQ existentes no mercado são ainda altamente tóxicas. A presente tese teve como objetivo desenvolver uma formulação PQ mais segura com a incorporação de LAS como antídoto, preservando a atividade herbicida. Para atingir estes objetivos a tese está estruturada em três estudos, com a finalidade de avaliar i) a segurança da formulação em ratos Wistar e a manutenção da atividade herbicida, (ii) a segurança da formulação para a microalga *Chlorella vulgaris*, e (iii) a toxicidade aguda e mecanismos de toxicidade da formulação, em comparação com o PQ.

Para esta finalidade, foi avaliada a toxicidade da mistura de Gramoxone® (formulação comercial contendo 20% de PQ) com LAS em ratos machos Wistar e a atividade herbicida foi testada em relva constituída por 50 % de *Poa pratensis* e 50 % de *Festuca arundinacea*. A taxa de sobrevivência do grupo PQ foi de apenas 40 %, enquanto no grupo que recebeu 125 mg/kg de PQ e 316 mg/kg de LAS foi de 100%. Além disso, o LD50 da formulação PQ + LAS é maior do que o LD50 do Gramoxone ® (34,86 mg/kg vs. 187,5 mg/kg, respetivamente). Incrivelmente, ambas as formulações de PQ, quer na

ausência ou quer na presença de LAS, possuem a mesma atividade herbicida contra as espécies testadas.

Com o objetivo de esclarecer os mecanismos envolvidos no efeito protetor do LAS, a formulação foi administrada a ratos Wistar machos por via oral, e estes foram sacrificados após 24 e 48h para avaliar as alterações de biomarcadores histológicos e bioquímicos. O tratamento com LAS causou uma redução significativa nos marcadores de peroxidação lipídica, de stress oxidativo e na ativação do fator nuclear kappa B (NF-kB) induzida pelo PQ no pulmão. Além disso, a eliminação de PQ foi maior no grupo PQ + LAS, a qual foi acompanhada pelo restabelecimento dos valores normais urinários de creatinina, proteínas e *N*-acetyl-β-glucosaminidase (NAG).

Devido ao risco de contaminação dos ecossistemas aquáticos aquando da aplicação dos herbicidas na agricultura, foi também objetivo desta tese avaliar a segurança desta nova formulação para os produtores primários, como a microalga *Chlorella vulgaris*. O PQ inibiu significativamente o crescimento *C. vulgaris*, um efeito que também foi prevenido significativamente pelo LAS, tanto na proporção utilizada na formulação [1:2 (mol/mol)], como na proporção de 1:8, onde foi atingida a proteção máxima.

Em conclusão, a presente tese atingiu com êxito os objetivos propostos através do desenvolvimento de uma formulação de PQ que demonstrou ser: (i) mais segura para ratos Wistar, (ii) igualmente eficaz contra as ervas daninhas, e (iii) menos tóxica para as microalgas.

Palavras-chave: herbicida, paraquato, antídoto, acetilsalicilato de lisina, algas, *Chlorella vulgaris*.

OUTLINE OF THE THESIS

The present thesis is structured in 8 Chapters:

Chapter I

In Chapter I, a general introduction of the state of art of the key topics within the thesis is presented. Once PQ has been studied since 1962, there is a huge number of publications concerning this herbicide. The introduction focused on the main characteristics of PQ as an herbicide, the mechanisms of toxicity, current and future treatments, acute and occupational poisoning. Considerable space is dedicated to the modifications applied to the formulations in the past few years. A critical thinking was provided along this chapter.

Chapter II

The Chapter II is divided in two review papers and one commentary. In Review I the several antioxidant mechanisms of salicylates are reviewed. A critical thinking of the current PQ-related research is provided in Commentary I. The most recent evidences linking pesticide chronic exposure with neurodegenerative diseases such as Parkinson´s Disease, Alzheimer´s Disease and Amyotrophic Lateral Sclerosis are also reviewed in this chapter in Review II.

Chapter III

The Chapter III comprises the general and specific objectives of the thesis.

Chapter IV

The Chapter IV is divided in three studies, corresponding to the original articles and describes the experimental work (materials, methods, results and discussion are presented in the form of manuscripts published, accepted or submitted for publication in peer-reviewed journals) in order to achieve the proposed aims.

Chapter V

In Chapter V the studies performed are discussed and integrated in a harmonized form.

Chapter VI

In this Chapter the main conclusions of the thesis are summarized.

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In this Chapter the prospects for future work are presented.

Chapter VIII

The references used in Chapter I and V are listed.

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LIST OF ABBREVIATIONS

AP-1, activator protein-1

ASA , acetylsalicylic acid

ASK, apoptosis signal-regulating kinase 1

Chlorella vulgaris, *C.vulgaris*

CK, creatinine kinase

COX, cyclooxygenase

DNA, deoxyribonucleic acid

GSH, reduced glutathione

GSSG, oxidised glutathione

HO-1, heme oxygenase 1

$HO^{\cdot}$, hydroxyl radical

H_2O_2, hydrogen peroxide

IL-1β, Interleukin-1 beta

IKKβ, IκB kinase β

LAS, lysine acetylsalicylate

LD50, lethal dose 50%

LPO, lipid peroxidation

MPO, myeloperoxidase

$NADP^+$, oxidized nicotinamide adenine dinucleotide phosphate

NADPH, reduced nicotinamide adenine dinucleotide phosphate

NAG, *N*-acetyl- *β*- glucosaminidase

NaSAL, sodium salicylate

NF-κB, nuclear factor kappa-B

Nrf2, NF-E2-related factor 2

$^{\cdot}NO$, nitric oxide

NOS, nitric oxide synthase

O_2, oxygen

$O_2^{\bullet-}$, superoxide anion

PD, Parkinson's Disease

PQ or PQ^{2+}, paraquat

$PQ^{\bullet+}$, paraquat monocation free radical

ROS, reactive oxygen species

SAL, salicylate

SN, *substantia nigra*

SNpc*, substantia nigra pars compacta*

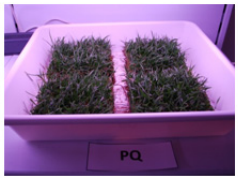
TGF-β, transforming growth factor

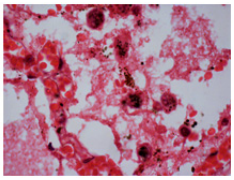
TNF-α, tumor necrosis alpha

Trx, thioredoxin

CHAPTER I

INTRODUCTION

Paraquat (1,1′-dimethyl-4,4′-bipyridylium dichloride; PQ) is the leading herbicide of the chemical family of bipyridylium quaternary ammonium herbicides. PQ was introduced in the market in 1962 by the Imperial Chemical Industries and since its introduction it has been used all over the world in more than 130 countries, although nowadays it is only registered and sold in 90 countries. In the past decade, the use of PQ has been controversial as it appears to be, in one hand, a cheap option for farmers to control weeds and, in another hand, extremely toxic to humans. For this reason, PQ has been banned from the European Union in 2007 and recently other countries have been restricting its use or applying a phase-out procedure. The fatalities due to PQ poisoning continue to be a heavy burden worldwide with a rate of fatalities between 30-80% (Bertolote et al., 2006; Lee et al., 2009b). The majority of the cases are suicide attempts followed by accidental poisonings and rarely by occupational exposure. The higher incidence of PQ poisonings occurs mainly in Asiatic countries such as Sri-Lanka (Eddleston et al., 2005; Senarathna et al., 2012), Indonesia, Malaysia, South Korea and China (Yin et al., 2013). China is currently the largest producer and consumer of PQ with an annual production of nearly 80,000 tons (Yin et al., 2013). However, the Chinese government is also following the same trend of other Asiatic countries by restricting the registration of new products containing PQ and demanding the complete withdraw of the herbicide from Chinese market until 2016. The obvious reasons are the extreme toxicity and lack of effective treatment measures. Despite the human poisonings, pesticide industry pressure and farmers demand for PQ are the reasons why this herbicide is still in the market in countries with severe regulatory systems such as USA, Canada, Australia, Japan, and New Zealand.

PQ presents exceptional agricultural properties with a very competitive price. PQ is a fast-acting, non-selective, contact herbicide which means that only the green part of the plants receiving the herbicide is affected. PQ is used in several varieties of crops including cereals, peanuts and cotton in North America; rice in China; coffee in Brazil; and oil palm in Malaysia. Furthermore, due to the nature of PQ residues in soils, the major part (some 99.99%) of PQ application that reaches the soil within the typical Good Agricultural Practice is strongly adsorbed to soils of a wide variety of textures (Bromilow, 2004). More relevant to environmental conditions is its rapid photodegradation on surfaces exposed to light, photolysis in aqueous solution, and biodegradation by soil microorganisms to non-toxic metabolites and ultimately to carbon dioxide (Roberts et al., 2002). PQ deactivation allows the sow of the crops almost immediately into treated soil without risk of phytotoxicity (Bromilow, 2004). This last property is particularly important as it limits

movement by leaching or in surface run-off, therefore restraining the ecotoxicological effects of PQ.

As a desiccating agent, PQ inhibits photosynthesis by intercepting the electron flow from photosystem I and shunting the electron transport chain. By accepting one electron, PQ is reduced to the PQ monocation free radical ($PQ^{\bullet+}$), which is rapidly reoxidised by O_2 to PQ^{2+}, initiating a series of reactions leading to production of superoxide anion ($O_2^{\bullet-}$) with subsequent cell membrane disruption and plant death. The mechanism of PQ phytotoxicity is transversal to other organisms, vegetal or mammalian. Despite the evidences of the poor environmental persistency profile, concerns regarding the environmental fate of PQ are still an important issue. A review of the literature has shown that continued use of PQ under Good Agricultural Practice conditions will have no detrimental effects on either crops or soil-dwelling flora and fauna (Roberts et al., 2002). However, even small amounts of herbicide reaching the aquatic environment can still be toxic. PQ affects the growth of different microalgae species from phytoplankton, which are the primary producers responsible for the maintenance of the equilibrium of the aquatic ecosystems, production of oxygen and organic substances, and therefore adverse effects of pollutants on these organisms may have reflexes in higher trophic levels (Carr et al., 1986; Ibrahim, 1990; Ma et al., 2004; Ma et al., 2006; Ma et al., 2002; Saenz et al., 1997).

The major burden concerning PQ use is undoubtedly its extreme mammalian toxicity. There are no widely accepted guidelines on treatment of patients with PQ self-poisoning and the treatment varies from supportive care alone to various combinations of immunosuppressors, antioxidant therapy, haemoperfusion and haemodialysis (Dinis-Oliveira et al., 2008b). However, the overall mortality remains >50% in centers routinely practicing such intensive measures. PQ poisoning results in toxicity in most organs but the toxicity is particularly severe in the lung (Smith and Heath, 1975). PQ pulmonary concentrations can be 6 to 10 times higher than those in the plasma, and the compound is retained in the lung even when blood levels start to decrease. Early experiments demonstrated that the accumulation of PQ into rat lung slices occurred against a concentration gradient (Rose et al., 1974). PQ structural similarities to the endogenous polyamines, putrescine and cadaverine led to the discovery of the putative lung transporter involved in PQ translocation, the polyamine uptake system. Therefore, PQ is mistakenly accumulated into the lung, especially in the alveolar type I and II pneumocytes and in the Clara cells, through this transport pathway (Smith, 1987).

Acute PQ poisonings are mostly due to ingestion of the concentrated liquid herbicide formulations and the severity of intoxication depends on the quantity of PQ ingested. The

symptomatology of PQ poisonings can be divided into three different presentations: asymptomatic or mild toxicity (<7.5 mL of 20% (m/v) concentrate), moderate to severe (7.5–15 mL of 20% (m/v) concentrate) and severe acute fulminant toxicity (>15 mL of 20% (m/v) concentrate). The moderate to severe poisoning culminates in pulmonary fibrosis, which progresses after a few days to a few weeks. There are three distinct phases in the progression of severe toxicity: (i) first phase: nausea, vomiting, gastrointestinal irritation/corrosion, and characteristic lesions on the tongue; (ii) second phase: acute proximal tubular necrosis, renal failure, and hepatocelluar necrosis; (iii) third phase: delayed pulmonary fibrosis leading to a rapid development of refractory hypoxemia, resulting in death. Patients surviving the early clinical stages generally show a period of improvement. However, in most cases this is merely the prelude to the late onset, which involves damage almost exclusively to the lungs. The lung pathophysiology of PQ is characterized by an early destructive phase (1-3 days) with extended alveolitis, pulmonary edema, and infiltration of inflammatory cells, followed by a final proliferative phase (where fibroblasts proliferate, collagen is deposited, and pulmonary fibrosis occurs).

The molecular mechanisms of PQ-induced toxicity in peripheral organs in humans are well understood. It is generally accepted that the primary reaction in the mechanism of PQ toxicity is associated with its capacity to undergo redox-cycling and subsequent generation of reactive oxygen species (ROS). PQ is metabolized by several intracellular enzyme systems [NADPH-cytochrome P450 reductase, xanthine oxidase, NADH-ubiquinone oxidoreductase and nitric oxide synthase (NOS) to the $PQ^{\bullet+}$ and reoxidized to PQ^{2+}, leading to the generation of $O_2^{\bullet-}$]. This then sets in the well-known cascade leading to the production of other ROS, mainly hydrogen peroxide (H_2O_2) by dismutation of $O_2^{\bullet-}$, and hydroxyl radical ($HO^{\cdot}$), through the Fenton or the Haber-Weiss reactions, with consequent cellular deleterious effects (Smith, 1987). The oxidative stress triggers and impairs several pathways which are involved in PQ deleterious effects. PQ induces a decrease in the ratio of reduced and oxidized nicotinamide adenine dinucleotide phosphate ($NADPH/NADP^+$), leading to inhibition of oxidized glutathione (GSSG) reduction to reduced glutathione (GSH) and therefore to detoxification of H_2O_2 by glutathione peroxidase; and inhibition of the pentose-phosphate shunt (Keeling and Smith, 1982). Consequently, there is a decrease in the intracellular antioxidant defenses as a result of the continue oxidation of GSH to GSSG and formation of protein mixed disulfides (Keeling et al., 1982). Electrophilic free radicals are capable of abstracting allylic hydrogen atoms from membrane-associated polyunsaturated fatty acids, resulting in alterations of membrane structure and ultimately, lipid peroxidation (LPO). *In vivo* studies have shown that PQ can cause lung protein oxidation assessed by carbonyl groups formation (Dinis-

Oliveira et al., 2006a; Dinis-Oliveira et al., 2006c), and DNA oxidation and fragmentation (Dinis-Oliveira et al., 2007a; Dusinska et al., 1998). PQ causes lung apoptosis by cytochrome c release, increase of caspase-3 and -8 activity, decrease of caspase-1 activity, and increase of p53 and activator protein-1 (AP-1) expression, resulting in DNA fragmentation (Dinis-Oliveira et al., 2007a). Indeed, PQ has been shown to cause mitochondrial damage (Czerniczyniec et al., 2011; Czerniczyniec et al., 2013) by inhibiting the complexes I and III, although this concept is not consensual between authors (Castello et al., 2007; Cocheme and Murphy, 2008). Release of cytochrome c and activation of caspase-9 was shown to be associated to the induction of Bcl-2 family members such as Bak, Bid, BNip3, and NOXA (Fei and Ethell, 2008; Fei et al., 2008). More recently, it was suggested that PQ-induced alveolar epithelial cell apoptosis is mediated through the NF-E2-related factor 2 (Nrf-2)-regulated mitochondrial dysfunction and endoplasmic reticulum stress (Chen et al., 2012). Moreover, Niso-Santano *et al.* investigated the role of the transcription factor Nrf2, a master regulator of cytoprotective genes, and its target thioredoxin (Trx), which binds and inhibits apoptosis signal-regulating kinase 1 (ASK1). PQ induced a dose-dependent decrease in Trx levels correlated with a major increase in phosphorylated ASK1, suggesting that Nrf2/Trx is crucial in PQ-induced apoptosis (Niso-Santano et al., 2011; Niso-Santano et al., 2010).

PQ-induced ROS also activate important inflammatory signalling pathways involving the nuclear factor kappa B (NF-κB) and AP-1 (Dinis-Oliveira et al., 2007a; Dinis-Oliveira et al., 2007b). The NF-κB family of transcription factors has an essential role in inflammation and innate immunity. NF-κB exists as homo- or heterodimers and are retained inactive in the cytoplasm of most cells, bound to the inhibitory proteins, IκBs (Hoesel and Schmid, 2013). The proinflammatory cytokines (tumor necrosis factor [TNF-α], interleukin 1 beta [IL-1β]) and oxidative stress activate IκB kinase β (IKKβ) leading to the degradation of the NF-kB complexes thus preventing the translocation to the nucleus and transcription of the target genes (Rahman and Fazal, 2011). The expression of proinflammatory genes such as Intercellular Adhesion Molecule 1 (ICAM-1) by this mechanism mediates adhesion and transendothelial migration of leukocytes. A characteristic feature of PQ toxicity is the massive pulmonary infiltration of leukocytes, particularly neutrophils. Then, local activation of rapidly responding resident cells, primarily macrophages, also release several proinflammatory cytokines, including TNF-α, IL-1β, and chemokines such as IL-8 and macrophage inflammatory protein 2α (Fan et al., 2001). Alveolar macrophages secrete fibrogenic factors such as transforming growth factor (TGF)-β (Assoian et al., 1987) and gene expression of TGF-β is enhanced in the lungs after PQ exposure (Ishida et al., 2006). Activated macrophages in inflamed lungs in response to PQ exposure also

synthesize increased amounts of several other cytokines, including interleukin 1 alpha (IL-1α), IL-1β, platelet-derived growth factor, TGF-α, insulin like growth factor, TNF-α that mediate an enhanced fibroproliferative response .

Although PQ liability is more significant regarding accidental or deliberate ingestion, there is a growing apprehension concerning the prolonged effects of occupational exposure to PQ. The herbicide is slightly toxic by the dermal route since absorption through intact skin is minimal (0.03 μg/cm^2 over 24 h), and has low inhalation toxicity , as PQ has no appreciable vapor pressure and the aerosolized particles are larger than 5 mm and, therefore, non-respirable (Environmental Protection Agency, 1997). Whether occupational PQ exposure is associated with adverse respiratory health effects among farmers is still controversial (Cha et al., 2012; Dalvie et al., 1999; Senanayake et al., 1993). Furthermore, the few occupational studies performed have shown that even after a dermal or inhalation exposure during application, urine levels were either undetectable (Chester et al., 1993; Van Wendel de Joode et al., 1996) or below the limit of quantification before-, during- and after-PQ spray days (Lee et al., 2009a).

Besides the respiratory adverse effects, the current spotlight of research is the putative involvement of PQ in the etiology of Parkinson's Disease (PD) (Dinis-Oliveira et al., 2006b; Kamel, 2013; Tanner et al., 2011). The *in vivo* and *in vitro* experimental data have shown that PQ is able to reproduce some of the features of PD such as specific cell loss on *substantia nigra pars compacta* (SNpc), and accumulation and increased aggregation of α-synuclein in dopaminergic neurons of *substantia nigra* (SN), but does not induce clear motor deficits (Cicchetti et al., 2009). The mechanisms of PQ-induced neurotoxicity are not fully comprehended yet, but several pathways have been proposed: (i) induction of oxidative stress, (ii) microglia activation, (iii) mitochondrial dysfunction, (iv) apoptosis, (v) autophagy and inhibition of the ubiquitin- proteasome system, and (v) induction of synucleinopathy and tauopathy. On the other hand, despite some studies found an association between PQ and increased risk for PD, the Human data may be misleading since the epidemiological studies currently available have a number of limitations. In addition, survivors of PQ poisoning have never presented parkinson signs during either the acute phase or the recovery period (Brent and Schaeffer, 2011). This emerging issue is extensively discussed in the Chapter II of this thesis.

Taking into consideration of the above mentioned rationale, it is no surprise that research of new antidotes and new formulations of PQ is still arousing international interest. In fact, the treatment of PQ intoxications has been directed towards: (i) preventing the generation of ROS, namely by the effective control of iron distribution by desferrioxamine, (ii)

scavenging ROS though the maintenance of effective levels of antioxidants, such as vitamin E and N-acetylcysteine, (iii) repairing the oxidative lesions, particularly the maintenance of effective levels of glutathione by administrating N-acetylcysteine, (iv) reducing inflammation by dexamethasone, methylprednisolone and cyclophosphamide, and (v) induction of glycoprotein-P by dexamethasone (Dinis-Oliveira et al., 2006a; Dinis-Oliveira et al., 2006c; Mitsopoulos and Suntres, 2011; Suntres, 2002; Suntres and Shek, 1995; Yeh et al., 2006). Other experimental pioneer antidotes include inhibitors of the angiotensin-converting enzyme (Ghazi-Khansari and Mohammadi-Bardbori, 2007; Ghazi-Khansari et al., 2007), mechanic ventilation with nitric oxide ($^{\cdot}$NO), propofol (Ariyama et al., 2000), edaravone (Zhi et al., 2011), inhibitors of quinone Oxidoreductase 2 (Janda et al., 2013), chitosan oligosaccharide (Yoon et al., 2011), naringin (Chen et al., 2013), bosentan (Zhang et al., 2013), and rapamycin (Lorenzen et al., 2010). However, neither of the proposed treatments conferred full survival after a LD50 dose was administered nor the schedule of administrations was consistent since some of the compounds were used prior to PQ poisoning (Chen et al., 2013; Mitsopoulos and Suntres, 2011; Yoon et al., 2011).

So far, only salicylates, sodium salicylate (NaSAL) (soluble form of salicylic acid) and lysine acetylsalicylate (LAS) were able to grant 100% survival to rats intoxicated with PQ (Dinis-Oliveira et al., 2009; Dinis-Oliveira et al., 2007a; Dinis-Oliveira et al., 2007b). NaSAL (200 mg/kg) decreased the PQ-induced lung toxicity through an effective inhibition of pro-inflammatory factors such as NF-κB, scavenging of ROS, inhibition of myeloperoxidase (MPO) activity, inhibition of platelet aggregation and apoptosis (Dinis-Oliveira et al., 2007a; Dinis-Oliveira et al., 2007b). LAS is the water-soluble form of acetylsalicylic acid (ASA) available in hospitals for parenteral administration, and therefore the subsequent studies were performed with this drug and also led to a successful outcome (Dinis-Oliveira et al., 2009). ASA is one of the most commonly used drugs worldwide with emerging novel clinical uses, well beyond the classical mechanism of non-steroidal anti-inflammatory drugs action: inhibition of prostanoids formation, which is achieved by inhibition of cyclooxygenase (COX). Since ASA is markedly de-acetylated to salicylate (ionic form, SAL), it could be assumed that the pharmacological effects of ASA are mainly mediated by its metabolite, given that both drugs exhibit similar anti-inflammatory activities *in vivo.* Nevertheless, SAL is a weak COX inhibitor due to the lack of the acetyl group but both drugs are strong inhibitors of NF-κB and AP-1 (Kopp and Ghosh, 1994; Whittle et al., 1980). Even though both salicylates have overlapping activities, ASA has other unique properties such as the induction of ferritin, lipoxins, NO,

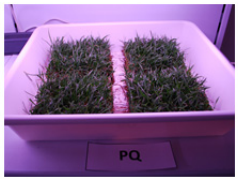
heme oxygenase 1 (HO-1), and inhibition of NAD(P)H oxidase activity. On the other hand, SAL strongly scavenges $HO^{\cdot}$ and chelate transition metals (Baltazar et al., 2011).

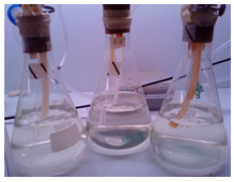
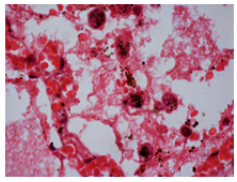
Despite the promising results, over the last few years there has been increasing pressure from government and regulatory authorities to develop formulations which have less impact on the environment and are safer for both applicators, manufacturers or people that accidentally or intentionally ingest those products. PQ producers also followed this trend to modify the formulation in order to reduce its toxicity. Firstly, a blue dye, a stenching agent and an emetic were added to the liquid concentrate in the late 1970s (Sabapathy, 1995). Gramoxone INTEON® is the most recent PQ formulation specifically developed to decrease toxicity through a reduction in the amount of PQ absorbed from the gastrointestinal tract following ingestion (Heylings et al., 2007). In order to achieve that goal, the new formulation included three novel components: (i) an alginate that immediately gels when entering the acidic environment of the stomach; (ii) an increase in the amount of emetic to induce vomiting more quickly; (ii) and an osmotic purgative (magnesium sulphate) to speed its elimination from the small intestine, the main site of its absorption. The survey realized after the introduction of INTEON® in Sri Lanka demonstrated that self-poisoning with this herbicide was associated with a significantly reduction of the mortality rate compared to the standard formulation (from 27.1 to 36.7%: $p<0.002$) (Wilks et al., 2008). However, years later a further assessment showed that this effect was not significant. The authors believe that this may be partly due to the large number of patients in whom PQ concentrations were too low for analytical confirmation of the formulation (Wilks et al., 2011).

The present work intends to implement a first principle of poisoning prevention: to change the formulation of the marketed product in an attempt to reduce human toxicity. If PQ use is to continue, and it seems that it will in many countries for the foreseeable future, then the answer must be to include an antidote to the formulation, as LAS, use more dilute PQ preparations as the primary product for agricultural use or make it more difficult to assess.

CHAPTER II

THEORETICAL BACKGROUNG

Review I

Antioxidant Properties and Associated Mechanisms of Salicylates

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Antioxidant Properties and Associated Mechanisms of Salicylates

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Abstract: The pharmacological action of salicylates has been historically related to their ability to inhibit cyclooxygenases, thereby blocking the synthesis of prostaglandins and thromboxane A_2. On the other hand, several studies have suggested that salicylates have a multitude of cyclooxygenase-independent actions specially related with their antioxidant properties, which might contribute to the overall salutary effects of these compounds. Although salicylates are well-known antioxidants through their ability to scavenge hydroxyl radical, their antioxidant mechanisms of action have not been fully compiled and characterized. In this context, several mechanisms of action have been suggested, namely i) scavenging of hydroxyl radical and chelation of transition metals; ii) upregulation of nitric oxide; iii) increased synthesis of lipoxins; iv) inhibition of neutrophil oxidative burst; v) inhibition of NF-κB and AP-1 protein kinases; and vii) inhibiton of lectin-like oxidized LDL receptor-1. The newly discovered acetyl salicylic acid-triggered lipoxins probably play a key role in the maintenance of the oxidative stress balance. Furthermore, salicylates have shown to protect low-density lipoprotein from oxidation and elicit an inhibitory effect on the expression of lectin-like receptors on endothelial cells. This review aims to provide an overview of the various proposed antioxidant mechanisms of salicylates.

Keywords: Salicylates, oxidative stress, antioxidant mechanisms.

1. INTRODUCTION

Willow bark and other salicylate-containing plants have been used for pain relief, fever and chronic inflammatory diseases such as arthritis and rheumatism since ancient times. As pharmaceutical drugs, salicylates were first introduced as analgesic antirheumatic drugs in 1876. Despite its unquestionable beneficial effects in inflammatory disorders, salicylic acid (SA) (the first salicylate to be used therapeutically) elicited an unpleasant sweet taste and was often associated with stomach ulcers accompanied by nausea and vomiting. These adverse effects were partially attenuated with the synthesis of the SA derivative, acetylsalicylic acid (ASA). Acetylsalicylic acid, commercially known as Aspirin®, is the acetylated form of SA, with improved gastric tolerability and similar anti-inflammatory activity [1]. Indeed, ASA, at the turn of its 112th anniversary, is one of the most commonly used drugs worldwide with emerging novel clinical uses, well beyond the classical mechanism of non-steroidal anti-inflammatory drugs (NSAID) action: inhibition of prostanoids formation, which is achieved by inhibition of cyclooxygenase (COX) (also referred as prostaglandin H synthase) [2-3]. After oral administration of an analgesic dose (1g single dose) of ASA, 50% is immediately hydrolyzed (de-acetylated) to SA, to some extent pre-systemically in the gastrointestinal mucosa and the remaining 50% is hydrolyzed within 15 minutes after gastric and intestinal absorption [1] (Fig. **1**). The plasma half-life of SA is dose-dependent and ranges from 2 to 30 hours [4]. The metabolism of SA occurs mainly in liver, through glycine conjugation to salicyluric acid, by enzymatic hydroxylation to 2,5-dihydroxybenzoic acid (gentisic acid) and by glucuronidation to produce either salicyluric acid phenolic-, salicyl phenolic- or salicyl acyl-glucuronide (Fig. **1**) [5].

Since ASA is markedly de-acetylated to SA, it could be assumed that the pharmacological effects of ASA are mainly mediated by its metabolite, given that both drugs exhibit similar anti-inflammatory activities *in vivo* [6]. Nevertheless, SA has been found to be a relatively weak inhibitor of COX activity *in vitro*, despite being an effective inhibitor of prostanoids formation at the place of inflammation *in vivo* [6]. Presently, there is no agreement about the extent of the contribution of SA to ASA´s anti-inflammatory effects [7].

The inhibiton of COX activity is responsible for the historical clinical applications of salicylates as analgesic/antipyretic and anti-inflammatory agents [1]. Nowadays, ASA is still commonly used to treat pain and febrile disorders but its use as anti-inflammatory drug is mainly restricted to particular inflammatory diseases, such as Kawasaki disease [1]. COX-1 is expressed in platelets where it stimulates the synthesis and release of thromboxane A_2 (TXA_2) resulting in platelet aggregation and vascular smooth muscle contraction [8-9]. Thus, the ASA property of inhibiting COX-1 activity is strategically used as an antithrombotic feature to prevent acute occlusive vascular events including ischemic stroke derived from platelet aggregation [10]. Thus, low-dose therapy is worldwide recommended for the primary and secondary prevention of myocardial infarction and for the treatment of unstable angina, non-ST-segment myocardial infarction, and acute coronary syndrome [10]. Also, numerous studies suggest that ASA has a protective effect on vasculature in several models of pathological conditions, including hypertension [11], hyperglycemia [12] and hyperlipidemia [13].

Recent studies have suggested further cyclooxygenase-independent mechanisms of action of salicylates that might explain their pleiotropic functions [14]. Accumulating epidemiologic data demonstrated that ASA prevents the development of colon rectal cancer, and decreases the risk for Alzheimer´s disease and atherosclerosis [14-15]. The etiology of these diseases has been associated with oxidative stress, which is a condition that results from the imbalance between the generation of reactive pro-oxidant species [reactive oxygen species (ROS) or reactive nitrogen species (RNS)] and the antioxidant defenses, in favor of the former, potentially leading to oxidative-related damage [16]. Noteworthy, low or moderate production of ROS and RNS play a physiological role in several redox-responsive signaling pathways, for example in defense against environmental pathogens, regulation of vascular tone by nitric oxide (NO), regulation of cell adhesion, and apoptosis [17].

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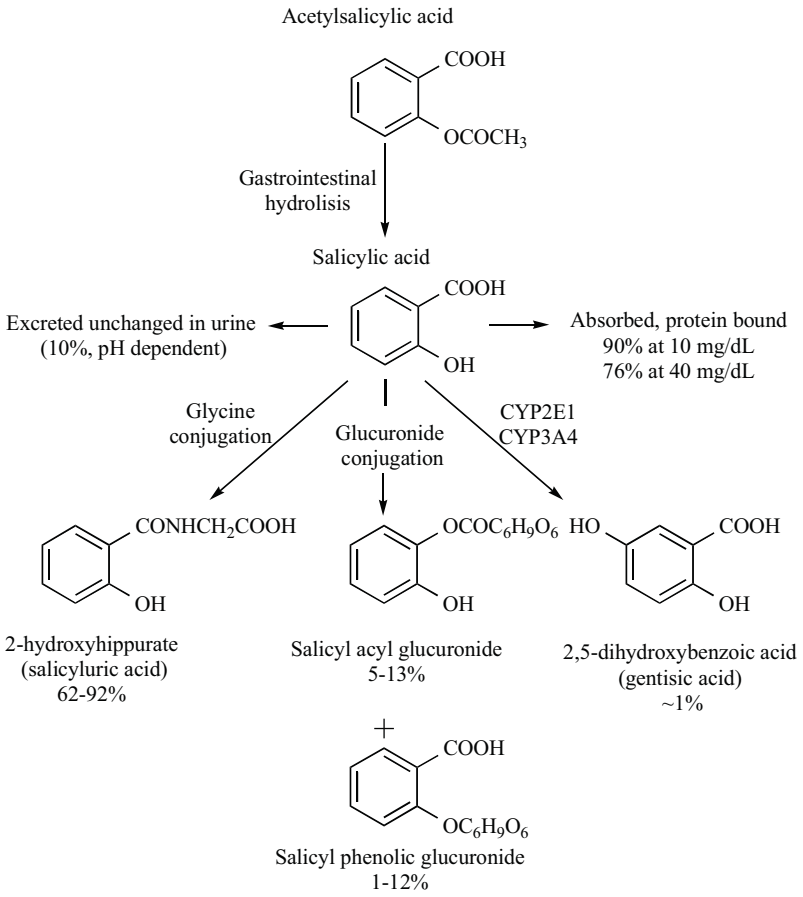

Fig. (1). Acetylsalicylic acid (ASA) metabolism pathways and relative concentrations of each metabolite following to salicylic acid clearance. ASA is rapid hydrolyzed to salicylic acid (SA) as the primary metabolite. The dominant metabolic pathway is conjugation with glycine to salicyluric acid (SUA). SA can also be conjugated with glucuronide to salicyl acyl glucuronide, salicyl phenolic glucuronide and hydroxylated to gentisic acid [1, 169].

Nevertheless, when these reactive species are sustainably produced in overwhelming amounts, may initiate a wide range of pro-oxidant reactions that result in damage of cellular macromolecules, including lipid peroxidation, protein nitration and oxidation, as well as DNA damage [17]. These events are known to be involved in the aging process [17] and in the pathogenesis of many degenerative diseases such as cancer [18-19], atherosclerosis [20] , arthritis [21], diabetes [22] and the neurodegenerative disorders Alzheimer' [23] and Parkinson´s diseases [24].

In the past few decades, a considerable and consistent amount of evidence has demonstrated that ASA and SA have antioxidant properties [11, 13, 25-26], though the mechanisms underlying these effects remain unclear. Firstly, it has been reported that salicylates comprise free radical-scavenging and iron chelation properties [27]. ASA was shown protects endothelial cells from oxidative stress mediated by hydrogen peroxide (H_2O_2) [28] and reduces vascular superoxide (O_2^{-}) production in both normotensive and hypertensive rats [11]. In a clinical study with normolipidaemic volunteers taking 300 mg of ASA, daily for two weeks it was shown that isolated low density lipoprotein (LDL) from these patients were more protected from oxidative modifications induced by ultraviolet C irradiation after the treatment [29]. In the several attempts to clarify the mechanisms responsible for the observed ASA-induced cytoprotection, it was noted that this drug had the ability to induce the expression of ferritin and hemeoxygenase-1 (HO-1), as well as to increase endothelial NO formation [30-33]. The discovery of new lipid mediators, the ASA-triggered lipoxins or 15-epi-lipoxin A_4 (ATL) [34] enlightened the molecular basis of additional antioxidant mechanisms suggested for ASA [35-36]. Indeed, ATL have shown the ability to increase NO synthesis through endothelial nitric oxide synthase [37], induce HO-1 expression [35] and inhibit peroxynitrite ($ONOO^{-}$) formation [38] as observed for ASA. Moreover, ASA downregulates transcription factors such as nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1), as well as the expression of lectin-like receptors (LOX-1) and several kinases that directly modulate signaling pathways linked to the immune and oxidative stress response [15, 39-40]. In addition, compelling evidence has shown that the vasoprotective effects of ASA might go beyond prostaglandin synthesis and be mainly due to its hypolipidaemic potential [41], inhibition of LDL oxidation [42] and improvement of endothelium function [43] mediated by ASA antioxidant properties.

Taken together, these data support the hypothesis that ASA possesses antioxidant activity by scavenging of hydroxyl radical and chelation of transition metals, stimulation of NO synthesis, increased expression of lipoxins, inhibition of neutrophil oxidative burst, inhibition of NF-κB, AP-1 and protein kinases, and inhibiton of lectin-like oxidized LDL receptor-1. The aim of this review is to elucidate these various mechanisms of action by which salicylates exert their antioxidant activity.

1. CYCLOOXYGENASE-DEPENDENT ACTIONS

The classically attributed mechanism of action of NSAIDS consists on the inhibition of prostaglandin H synthase's COX activity, blocking the biosynthesis of prostaglandins [3, 8]. There are at least two distinct isoforms of this enzyme, designated COX-1 and COX-2 [44]. COX-1 is considered to be the constitutive isoform, ex-

pressed in most tissues where it plays a key role in platelet aggregation and gastric protection [44]. It is widely assumed that ASA causes an irreversible inhibition of COX-1 activity by acetylation of a critical serine (Ser-530) in the substrate channel of the COX-1 enzyme, limiting the access of arachidonic acid (the substrate) to the catalytic active site [3, 45]. The other isoform, COX-2, is induced during inflammation, wound healing, and neoplasia and is responsible for the synthesis of the majority of prostaglandins [44]. Nevertheless, this concept is evolving since its known that COX-2 is expressed constitutively in several tissues including brain [46], kidney [47] and arterial endothelial cells under steady-state shear stress [48]. ASA is a nonselective inhibitor of COX activity, although it seems to possess a higher affinity for COX-1 [7]. This ability is responsible for its recognized anti-platelet properties since COX-1 is constitutively expressed in platelets where it stimulates the synthesis and release of TXA_2 [8-9]. Furthermore, platelets are not capable of regenerating COX-1 since they lack the biochemical machinery necessary to its synthesis. Therefore, inhibition of the TXA_2 is maintained during platelets life-time (7 to 10 days) [49]. Moreover, ASA triggers the synthesis of a unique class of eicosanoids by acetylation of COX-2 and 5-lipoxygenase (5-LO), which may additionally contribute to the therapeutic impact of this drug (Fig. **2**) [34-36, 50] (Fig. **4**).

1.1. Increased Expression of Lipoxins

As described above, inhibition of prostaglandins and TXA_2 formation by ASA is achieved through irreversible inhibition of COX-1 by acetylation [3]. Nevertheless, acetylation of vascular COX-2 by ASA does not lead to inhibition of the enzyme, but rather switches the catalytic activity for conversion of arachidonic acid to intermediates of prostaglandines and TXA_2 into the production of 15R-hydroxyeicosatetraenoic acid (15R-HETE) [34]. 15R-HETE is released from endothelial and epithelial cells and is converted by leukocyte 5-LO to 15(*R*)-epi-lipoxin A_4 also referred as ASA-triggered lipoxin (ATL) (Fig. **2**) [34].

Lipoxins (LXs), produced by leukocytes during cell-cell interactions, are a distinctive class of endogenous lipid mediators with strong anti-inflammatory activity [51]. Inhibition of neutrophils' recruitment, and decrease in chemokine production by native lipoxin A4 (LXA_4), and its analogues, is mediated by the activation of the G protein-coupled lipoxin A4 receptor (ALXR) [52]. LXA_4 is capable of attenuating the chemotaxis, adhesion and transmigration of neutrophils and this ability to block endothelial cell-leukocyte interactions can be seen as a protective mechanism against reactive oxidants produced by activated leukocytes [53]. Furthermore, LXA_4 inhibits $O_2^{\cdot-}$ formation, selectively stimulates monocytes and macrophages in a nonphlogistic fashion leading to the phagocytosis of apoptotic polymorphonuclear cells (PMN) by monocyte-derived macrophages and blocks tumor necrosis factor alpha (TNF-α) actions [53-55].

ASA-triggered lipoxins and the endogenous LXA_4 have similar biological activities as both downregulate the neutrophils-mediated tissue damage [50]. Thus, ATL may account for the additional effects of ASA not related to the inhibition of the cyclooxygenase system, namely the antioxidant activity [36, 52, 56-57].

Recent studies have shown that the stable ASA-triggered lipoxin A_4 analog (15-epi-16-(para-fluoro)-phenoxy-lipoxin A_4) (ATL-1) potently promotes HO-1 *de novo* synthesis in a concentration and time-dependent manner (Fig. **2**) [35]. Also, in this study it was confirmed the involvement of ALXR since an antagonist of this receptor blocked the ATL stimulation effect on HO-1 expression. Moreover, HO-1 induced by ATL significantly inhibited the TNF-α-induced expression of the adhesion molecules, endothelial adhesion molecule 1 (E-selectin) and vascular cell adhesion molecule-1 (VCAM-1). This phenomenon was corroborated by the fact that a HO-1 inhibitor, tin-protoporphyrin (SnPP), reversed the LXA_4-induced inhibition of VCAM [35]. HO-1 is also a downstream target of NO [30, 32]. Thus, the induction of HO-1 expression by ATL-1 might also be mediated *via* eNOS and NO [37]. Of note, ATL-1 increases plasma NO with subsequent inhibition of leukocyte trafficking *via* eNOS and inducible nitric oxide synthase (iNOS), illustrated by experiments with knockout mice for these enzymes [37].

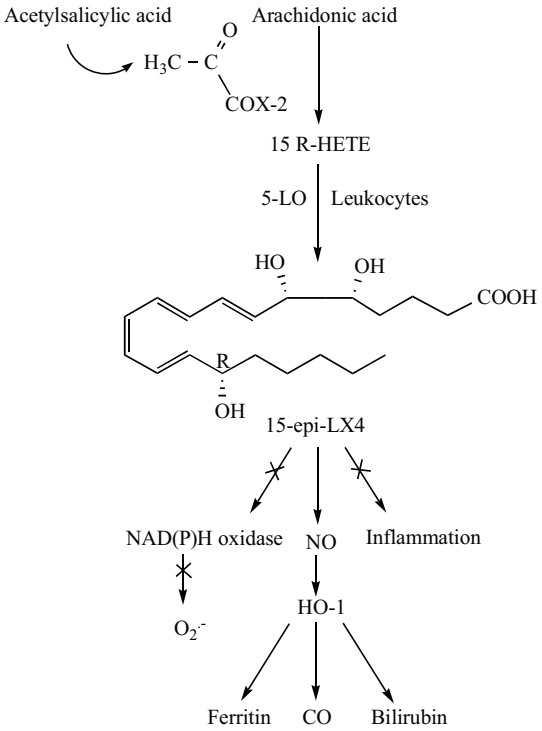

Fig. (2). Induction of NO synthesis and expression of HO-1 by acetylsalicylic acid and aspirin-triggered lipoxins. Bilirubin and ferritin act as potent antioxidants and CO as an anti-inflammatory mediator in vascular and nonvascular cells. ALT are key mediators in the inflammatory response and oxidative stress. 15-epi-LXA_4, aspirin-triggered carbon-15 epimers lipoxins; 15R-HETE, 15R-hydroxyeicosatetraenoic acid; COX-2, cyclooxygenase-2; CO, carbon monoxide; HO-1, heme oxygenase 1; 5-LO, 5-lipoxygenase; NAD(P)H oxidase, reduced nicotinamide adenine dinucleotide phosphate oxidase; NO, nitric oxide; $O_2^{\cdot-}$, superoxide radical.

In a model of stroke-prone spontaneously hypertensive rats (SHRSP), chronic treatment with ASA improved eNOS expression and NOS activity in the arteries of salt-loaded SHRSP, in association with a significantly increase of ATL levels [58]. As other studies demonstrated [34, 37], neither SA nor indomethacin improved NO release or triggered ATL synthesis in salt-loaded SHRSP [58]. Collectively, it seems that ASA triggers the synthesis of ATL, which contributes to increase NO synthesis and the consequent expression of the antioxidant protein HO-1, hence inhibiting leukocyte-endothelium interactions during acute inflammation. These observations provide evidence that there is an antioxidant synergism between two pathways, the lipoxins and the HO-1,NO being the linking element [59].

The stable ASA-triggered lipoxin analog abolishes NAD(P)H oxidase-dependent ROS generation in a concentration and time-dependent manner in endothelial cells and requires ALXR signaling (Fig. **2**) [36]. Endothelial NAD(P)H oxidases are important sources of reactive oxygen species. It is assumed that the sustained activation of these enzymes results in a variety of intracellular signaling events that may lead to a dysfunction of the endothelium, proliferation of vascular smooth muscle cells, expression of pro-inflammatory and pro-apoptotic genes and remodeling of the ex-

tracellular matrix [60]. The prototypical NAD(P)H oxidase is the isoform found in neutrophils, which is constituted by four major subunits: plasma membrane cytochrome b558, comprising $gp91^{phox}$ and $p22^{phox}$, and two cytosolic components $p47^{phox}$ and $p67^{phox}$ [60]. The pathophysiological increase in NAD(P)H oxidase activity and expression can be triggered by a number of growth factors (e.g. transforming growth factor and platelet-derived growth factor), cytokines (e.g. TNF-α and IL-1), laminar shear stress, hyperinsulinemia, hyperglycemia, and G protein-coupled receptor agonists (e.g. serotonin, thrombin, endothelin and angiotensin II) [61]. Despite Wu and colleagues [11] have suggested a novel antioxidant pathway of ASA by inhibiting NAD(P)H oxidase, which was corroborated by other authors [62], the underlying molecular mechanism was not elucidated. In human endothelial cells exposed to high glucose concentrations, Dragomir and co-workers [62] demonstrated that ASA-induced reduction of protein kinase C activation is a key event for NAD(P)H oxidase activation [63]. As mentioned above, ASA-triggered lipoxin analog abolishes NAD(P)H oxidase-dependent ROS generation through ALXR signaling. Furthermore, ATL-1 inhibits the $p47^{phox}$ phosphorylation and subsequent translocation and stable binding to $p22^{phox}$ on the endothelial cells membrane. This is a key event that precedes NAD(P)H oxidase activation and ATL-1 seems to interfere on the enzyme assembly, probably because it modulates the $p47^{phox}$ phosphorylation status [36, 60].

Angiotensin II (Ang II) is a strong inducer of NAD(P)H oxidase and therefore stimulates a number of biological responses *via* redox-sensitive mechanisms, including the pro-inflammatory transcription factor NF-κB [61]. ATL-1 significantly inhibits Ang II-induced NF-κB activation and consequently this effect will reduce the synthesis of pro-inflammatory cytokines, chemokines and inducible enzymes [36]. Similarly, lipopolysaccharide (LPS) challenged PMN and mononuclear leukocytes evoke nuclear accumulation of NF-κB/p65 and AP-1/c-fos [38]. However, when these cells are incubated with LXA_4/ATL analogs, the mobilization of these transcription factors to the nucleus is inhibited as well [38]. In the same experimental model, LXA_4/ATL analogs markedly attenuated $ONOO^-$ formation, IL-8-mRNA expression and IL-8 release possibly *via* inactivation of NF-κB and AP-1 [38]. Peroxynitrite is formed in the reaction of NO with $O_2^{\cdot-}$ and is predominantly released by inflammatory cells during several inflammatory pathologies. Peroxynitrite is highly reactive towards all classes of biomolecules including nucleic acids, lipids and proteins. It has been suggested that $ONOO^-$ is involved in cell signaling pathways that modulate leukocyte trafficking through induction of cytokines such as IL-6 and TNF-α and up-regulation of surface expression of CD11b/CD18 and consequently increase in PMN adhesion to endothelial cells [64-65]. ATL-1-mediated inhibition of NAD(P)H ultimately reduces $O_2^{\cdot-}$, which eventually can decrease $ONOO^-$ by withdrawing $O_2^{\cdot-}$ from the site of reaction with NO [36]. Furthermore, this hypothesis may be an additional explanation for the observed increase of NO evoked by ASA and 15-epi-lipoxins [37, 66].

Chiang and colleagues [50], reported that daily treatment with low dose (81 mg) of ASA triggers the formation of ATL in healthy individuals *in vivo*. The detectable amount of lipoxins generated is sufficient to evoke the anti-inflammatory actions [34] and up-regulation of antioxidant mediators such as NO and HO-1 [35, 37]. Therefore, local production of ATL may also account for the general pharmacological actions of ASA.

1.2. Inhibition of Neutrophil Chemotaxis

As mentioned above, salicylates are potent inhibitors of platelet aggregation. Platelets function has been exclusively associated to the maintenance of hemostasis and platelet-derived growth factors [67]. However, several lines of evidence support the important role of platelets in inflammation [67-68]. During the inflammatory process, platelets undergo chemotaxis, release proinflammatory cytokines and adhesive proteins, and recruit neutrophils [69]. Indeed, a significant role of platelets in the inflammatory process is their ability to promote neutrophils rolling, leading to its firm adhesion to the endothelium with further diapedesis [69]. Chronic inflammation

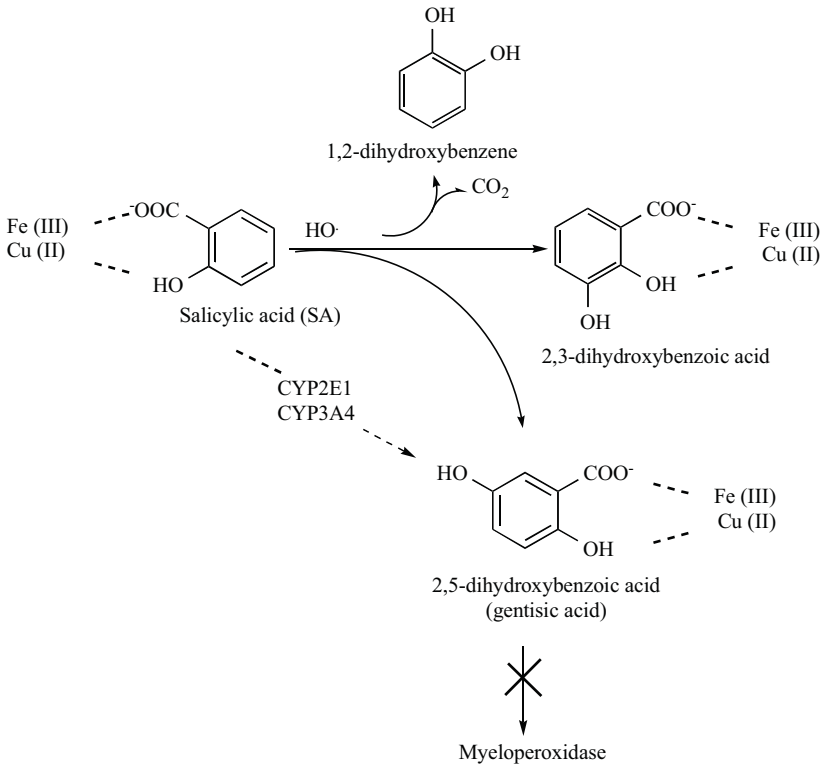

Fig. (3). Hydroxylation products of salicylic acid and associated biological activities.

mediated through platelets also leads to oxidative injury of the endothelium and the inflamed tissue, hence there is a linkage among platelets and inflammation, thrombosis, and atherogenesis [67]. Thus, the antiplatelet beneficial effects of ASA, preventing atherothrombotic events (e.g. arterial thrombosis), acute myocardial infarction and cerebrovascular diseases, might be further due to its inhibition of the proinflammatory cascade and oxidative stress [10] (Fig. **4**).

2. SCAVENGING OF HYDROXYL RADICAL AND CHELATION OF TRANSITION METALS

Among ROS, hydroxyl radical ($HO^{\cdot}$) is the most reactive radical known, as it can nonspecifically oxidize all classes of biological macromolecules including lipids [70-71], proteins, and nucleic acids [72]. The *in vivo* antioxidant efficacy of $HO^{\cdot}$ scavengers will depend on the rate constant for reaction with $HO^{\cdot}$, their metal-binding capacity, the geometry of the metal complex formed as well as the availability of transition metals in solution directly correlated to the physiological levels of phosphate and bicarbonate [73]. The $HO^{\cdot}$ generation is site-specific, frequently at sites of inflammation, for example in the inflamed rheumatoid joint where the pH is acidic [74]. At this acidic environment, SA has two effective binding sites, referred as a bidentate chelation site [75]. The transition metals, Fe(III) and Cu(II) bind to these sites forming the Fe(III) and Cu(II)-salicylate complexes (Fig. **3**) [75]. Thus, chelation of transition metals at the sites of inflammation decreases the probability of $HO^{\cdot}$ generation through the Fenton reaction (Equation 1) [27, 76].

$$H_2O_2 + Fe^{II}(L)_n = Fe^{III}(L)_n + HO^{-} + HO^{\cdot} \quad (1)$$

Besides its chelation properties, it is also well known that ASA and mainly its SA metabolite strongly scavenge $HO^{\cdot}$ [77]. The major evidence for this activity is the ability of ASA and SA to undergo aromatic hydroxylation by reacting with $HO^{\cdot}$ (Fig. **3**) [27, 78]. Accordingly, the application of SA as a chemical trap for $HO^{\cdot}$ represents a simple and convenient technique to study the *in vivo* and *in vitro* production of free radicals [77, 79]. The major hydroxylation metabolites resulting from $HO^{\cdot}$ attack on SA, under physiological conditions, are 2,5-dihydroxybenzoic acid (2,5-DHBA; gentisic acid), 2,3-dihydroxybenzoic acid (2,3-DHBA) and, to a smaller extent, the catechol, 1,2-dihydroxybenzene (Fig. **2**). Other minor products include 2,4- and 2,6-DHBA [80]. Both isomers, 2,5-DHBA and 2,3-DHBA, can be measured as markers of $HO^{\cdot}$ formation by high-performance liquid chromatography with electrochemical detection [77, 81]. 1,2-dihydroxybenzene and 2,3-DHBA have not been reported as products of enzymatic metabolism by cytochrome P450 in contrast to 2,5-DHBA. Thus, 2,3-DHBA is considered a specific marker of *in vivo* $HO^{\cdot}$ production [82] and it has been used, for instance, to study postischemic oxidative injury in isolated tissues [83-84]. In addition, 2,3-DHBA is a potent iron chelator (Fig. **3**) [85] and, as it was referred before, iron is required for $HO^{\cdot}$ generation *via* Fenton reaction.

Importantly, when used for the treatment of inflammatory processes, ASA and its metabolite SA originate sufficiently high concentrations at sites of inflammation [86]. Thus these drugs are considered better *in vivo* $HO^{\cdot}$ scavengers than other several well established antioxidants (e.g. ascorbate, GSH and cysteine) not only because of the site-specific location, iron-binding ability but also due to the high reaction rate of $HO^{\cdot}$ with ASA (3.6×10^{10} M^{-1} sec^{-1}) and SA ($6\text{-}10\times10^{9}$ M^{-1} sec^{-1}) [25].

3. SUPEROXIDE DISMUTATION

Jay and co-workers [87] demonstrated that the salicylate-iron complex has superoxide dismutase (SOD) activity, confirmed by the inhibition of the reduction of nitroblue tetrazolium at pH 7.8 in a xanthine-xanthine oxidase system (Fig. **4**). In these experiments it was shown that iron and the salicylate-iron complex have also xanthine oxidase inhibitory properties [87], which represents another antioxidant mechanism for salicylates. On the other hand, the authors also observed that during the dismutation reaction, the formation of H_2O_2 and simultaneously the reduction of Fe(III) of the complex to Fe(II), may occur, leading to the generation of $HO^{\cdot}$ [87].

4. NITRIC OXIDE-DEPENDENT ACTIONS

Nitric oxide is involved in numerous biological processes including inflammation, neurotransmission, gene regulation and vasodilation [88]. However, NO is also one of the proposed cytotoxic species produced by the immune surveillance system. At physiological concentrations, NO has also been shown to protect endothelial cells [89], fibroblasts [90], hepatocytes [91], and cardiomyocytes [92] from cytotoxicity produced by oxidative stress. NO is a potent terminator of radical chain propagation reactions during lipid peroxidation, as observed in model lipid systems [93], low density lipoprotein oxidation [94] and in cell cultures [91, 95]. On the other hand, at high concentrations or when it reacts with O_2^{-}, forming the highly reactive $ONOO^{-}$, it can responsible for cellular damage [88].

In the past few years, compelling evidence suggests that ASA increases NO formation and this pathway contributes to the prevention of endothelial injury in cardiovascular diseases [66]. It was demonstrated that the increase of NO synthesis is mediated through a stimulatory effect of ASA on eNOS since the inhibitor of NO synthase, L-N^{G}-monomethyl arginine (L-NAME), and the NO scavenger, PTIO, were shown to completely abolish cytoprotection by ASA [32] (Fig. **4**). Furthermore, ASA was shown to induce an immediate concentration-dependent NO release from porcine coronary artery, with a half maximal effective concentration of 50 nM, by a calcium-independent mechanism [66]. The concentration required to enhance NO release is within the range of concentrations achieved during low-dose regimens [96]. ASA may enhance accumulation of endothelial soluble guanylyl cyclase (cGMP) levels through a direct effect on eNOS and in a NO-dependent manner as both elevations of NO and cGMP were attenuated by L-NAME [32, 66]. Additionally, this effect appears to be due to a direct acetylation of eNOS. Given the rapid onset of its action on the enzyme activity, a genomic action of ASA seems unlikely, though ASA may also indirectly induce the expression of eNOS through the increased production of lipoxins (Fig. **2**). Other NSAIDs including SA, failed to enhance NO generation in endothelial cells, which demonstrates that the acetyl group in ASA is a structural requirement for NOS activation [32]. In addition, ASA analogs containing an acetyl group were capable of mimicking activation of isolated eNOS and the extent of NO release was strongly correlated with the acetylation potency of these compounds [66]. Currently, the molecular level at which eNOS acetylation occurs is not clear. Taubert and colleagues [66] have shown that ASA increases citrulline formation from L-arginine, and therefore it seems possible that ASA modulates eNOS post-transcriptionally by affecting its substrate accessibility, cofactors (e.g. NADPH or tetrahydrobiopterin) or protein-protein interactions [97]. It is noteworthy that NO and NO donors produced a very similar profile of endothelial protection evoked by ASA [98].

5. INDUCTION OF HEME OXYGENASE-1

HO-1 is an inducible isoenzyme responsible for the rate-controlling step of heme catabolism [99]. The highest HO-1 activity is found in the spleen, testes, and brain [100]. HO-1 is induced by a range of oxidative stress stimuli such as transition metals, LPS, hyperoxia, ischemia, NO, UV light and strongly by its heme substrate. These stimuli lead to the induction of HO-1 in tissues such as the heart, vasculature, liver and kidney, where under normal conditions, the enzyme has a lower activity [100].

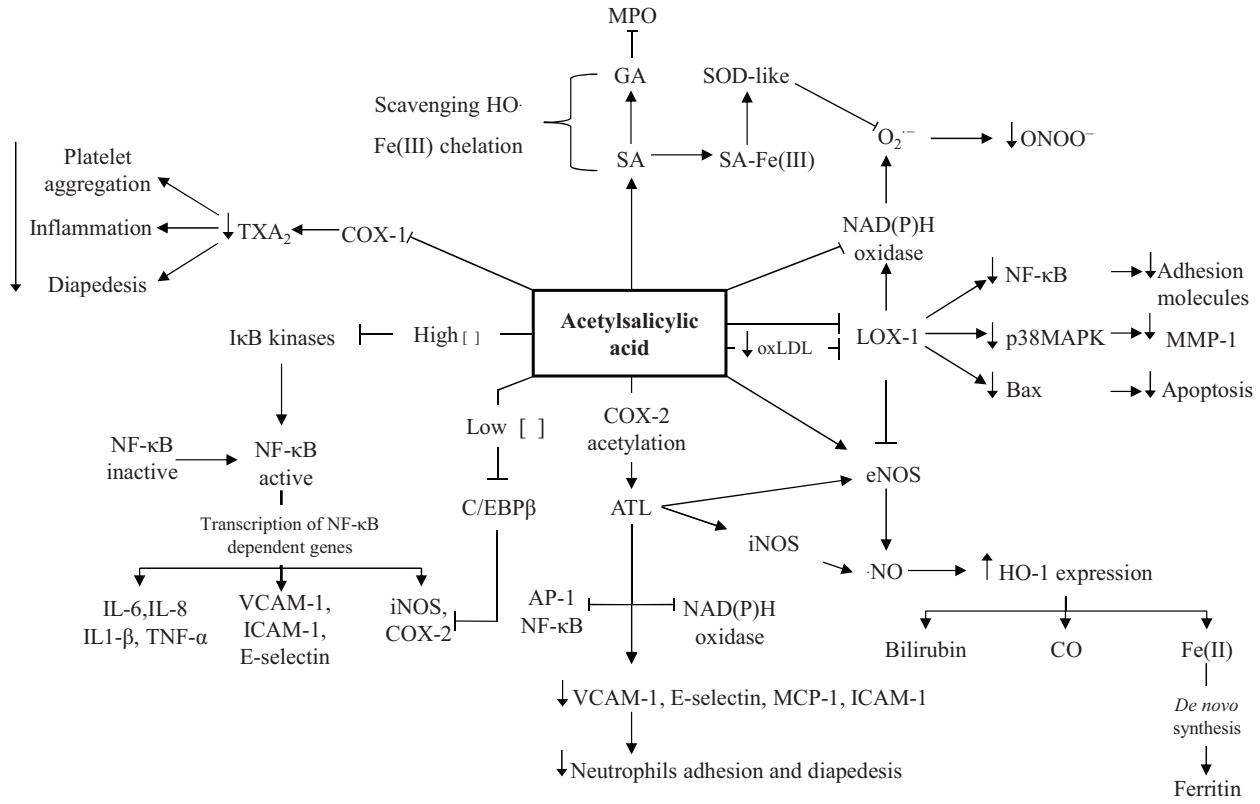

Fig. (4). Schematic overview of the potential antioxidant pathways of salicylates. Activation or induction (→); inhibition (⟞); AP-1, activator protein 1; ALT, acetylsalicylic acid-triggered lipoxins; Bax, bcl-2–associated X protein; CO, carbon monoxide; COX, cyclooxygenase; C/EBP β, CAAT enhancer binding protein beta; eNOS, endothelial nitric oxide synthase; E-selectin, endothelial adhesion molecule 1; Fe(II), ferrous ion; $HO^{\cdot}$, hydroxyl radical; HO-1, heme oxygenase 1; ICAM-1, inter-cellular adhesion molecule 1; IL, interleukin; iNOS, inducible nitric oxide synthase; LOX-1, lectin-like oxidized low-density lipoprotein receptor 1; MAPK, mitogen-activated protein kinase**;** MMP, matrix metalloproteinase; NADPH oxidase, reduced nicotinamide adenine dinucleotide phosphate oxidase; NF-κB, nuclear factor kappa B; NO, nitric oxide; $O_2^{\cdot-}$, superoxide radical; $ONOO^{-}$, peroxynitrite; oxLDL, oxidized low density lipoprotein; SA, salicylic acid; SOD, superoxide dismutase; TNF-α, tumor necrosis factor alpha; TXA_2, thromboxane A2; VCAM-1, vascular cell adhesion molecule 1.

Grosser and coworkers [33], proposed that the antioxidant profile of ASA is attributable to its ability to stimulate the expression and enzymatic activity of HO-1 in endothelial cells. In that study, the HO-1 induction and the endothelial protection was achieved at micromolar concentrations of ASA, which are within the plasma or tissue levels attained after oral therapy for cardiovascular diseases. Therefore, it could be assumed that HO-1 plays an important role in endothelial cells against oxidative stress injury in view of the fact that, in the presence of the HO-1 inhibitor tin-protoporphyrin (SnPP), the cellular protection afforded by ASA was abolished. Grosser and Schroder [32] demonstrated that the induction of HO-1 by ASA was NO-dependent since the inhibitor of NO synthase, L-NAME, abolished ASA-induced HO-1 activation. These findings are in agreement with prior studies supporting that NO is a downstream mediator of HO-1 [30] and that ASA elicits NO release from vascular endothelium at therapeutic concentrations [66] (Fig. **2**). Remarkably, these effects are specific to ASA and not induced by other NSAIDs such as diclofenac, indomethacin, or SA or by selective COX-2 inhibitors [31, 33]. On the other hand, NO and NO donors have been reported as inducers of HO-1 trough cGMP-dependent pathways [30]. Besides enhancing the expression of HO-1 at the mRNA level, NO was reported to increase the protein expression, the catalytic action and to prolong the half-life of HO-1 mRNA [101-102].

The activation of *HO-1* gene is considered an adaptive mechanism of cell survival since it has been described that overexpression of HO-1 provides cytoprotection in a variety of cell culture and animal models [103]. The HO-1 system is physiologically significant because it catalyzes the oxidation of the pro-oxidant heme to biologically active catabolites as (i) iron, a gene regulator; (ii) bilirubin, an antioxidant; and (iii) carbon monoxide (CO), a heme ligand [100].

The HO-1 product biliverdin is rapidly converted to bilirubin by biliverdin reductase. At micromolar concentrations, bilirubin exhibits a strong antioxidant activity by scavenging peroxyl radicals [104-105]. In accordance, in a model of H_2O_2-mediated toxicity to endothelial cells [33], it was shown that exogenous bilirubin, at physiological plasma levels, increased cellular resistance to oxidative damage. Other studies also demonstrated that bilirubin protects neuronal cells from oxidative stress [106]. Furthermore, HO-1, in cultured endothelial cells, inhibits the ability of proinflammatory cytokines such as TNF-α or interleukin-1-β (IL-1-β) to induce the expression of adhesion molecules, as the inter-cellular adhesion molecule-1 (ICAM-1), VCAM-1, and E-selectin, bilirubin being a key factor for this action [107]. Consequently, serum bilirubin has been considered a novel coronary heart disease risk marker with a strong inverse association [108]. Thus, the ASA induction of HO-1 and further increase in bilirrubin production may also contribute to explain the antioxidant and beneficial cardiovascular effects of this drug.

Heme oxygenase-derived CO exerts anti-apoptotic, anti-inflammatory, anti-platelet and vasodilation effects [109]. The other HO-1 metabolite, iron, leads to *de novo* synthesis of ferritin protein, an antioxidant mediator [110] (see below). Nonetheless, it is note-

worthy that free iron is a catalyst of Fenton reaction leading to generation of $HO^{\cdot}$ [111]. Thus, the induction of HO-1 may be one of the mechanisms underlying the observed induction of ferritin synthesis through ASA [31].

HO-1 is considered a promising target for the treatment of cardiovascular diseases, including atherosclerosis, and neurodegenerative processes such as Alzheimer and Parkinson's disease, because it can trigger a unique combination of protective actions on tissue and smooth muscle-relaxing effects. Thus it is conceivable that the antioxidant activity and antiatherogenic properties of ASA are at least partially attributable to the increase of HO-1 expression and ensuing formation of bilirubin and CO (Fig. **2**).

6. INDUCTION OF FERRITIN SYNTHESIS

Iron is an essential physiological metal and it is a required component of several enzymes (e.g. ribonucleotide reductase, xanthine oxidase, catalase, cytochrome P450) and oxygen binding molecules such as hemoglobin and myoglobin [112]. Despite its biological benefits, free iron might also have deleterious effects due to its ability of catalyzing the oxygen-dependent radical formation *via* Fenton reaction. Indeed, compelling evidences suggest that excess free iron is a risk factor for developing several types of cancer [113-115] and coronary diseases [116], raises the incidence of myocardial infarction [117] and promotes atherosclerosis [118-119].

Ferritin is a high molecular mass (450 kDa), multimeric protein constituted by 24 polypeptide chains of two types (Heavy or H chain, and Light or L chain), which form a cavity with a high capacity for storing iron (4,500 mol of iron/mol of ferritin) [120]. Ferritin plays a key role in iron metabolism since, additionally to its storage, is able to sequester free cytosolic iron, the highly toxic Fe(II) and transform it into the non- toxic Fe(III) as an inorganic complex [121]. Thus, ferritin removes the free metal ions from the site of radical formation and therefore prevents the oxidative stress cascade leading to tissue damage.

It is well established that ferritin synthesis is induced in response to an elevation of free iron serum concentrations through post-transcriptional mechanisms and increase in ferritin mRNA [122]. Furthermore, several findings support that oxidative stress stimulus applied to different types of cultured cells enhances ferritin synthesis, hence decreasing the accumulation of ROS [110, 121, 123-125]. As mentioned above, the increase in ferritin synthesis can be mediated by HO-1 as an adaptive response to oxidative stress [126]. Oberle and co-workers [31], showed, for the first time, that ASA (30-300 μM) increases ferritin synthesis in endothelial cells in a dose and time dependent manner. The stimulatory effect of ASA was limited to the translation level, given that ferritin mRNA synthesis was unaltered. In the same study [31], other NSAIDs such as SA, diclofenac, and indomethacin failed to up-regulate ferritin, which suggests that the acetyl group is a structural requirement to inactivate the ferritin repressor protein from the ferritin mRNA, allowing the translation of the protein.

Although ferritin expression was shown to increase in *in vitro* studies using endothelial cells exposed to ASA [31, 127], other authors reported contradictory results after treatment with ASA in humans. In an US elderly population [128] the use of ASA was associated with significantly lower serum ferritin levels, consistent with Milman and colleagues findings in a Danish population [129]. Fleming [128] hypothesized that this effect could be a consequence of lower body iron stores resulting from increased occult blood loss, an adverse effect of ASA. A further observation described an inverse association between ASA intake and serum ferritin which was more marked in the subgroup of diseased elderly subjects with underlying infection, liver disease or inflammation. Ferritin was described as an acute phase inflammatory protein, inducible by interleukin-1-beta (IL-1-β) and tumor necrosis alpha (TNF-α) [130]. The inhibition of cytokine-mediated synthesis and secretion of ferritin from the liver is a possible mechanism to explain the observed effects of continuous ASA intake leading to the assumption that ferritin levels in these cases are unlikely to be related to iron stores. Although the existence of some conflicting results in humans, it can be hypothesized that ASA induction of ferritin in endothelial cells plays a direct vasoprotective effect and this mechanism is likely to attenuate the activation of both injury and pathophysiological signaling pathways associated with the development of vascular disease.

7. INHIBITION OF MYELOPEROXIDASE

Oxidative burst results from the release of oxidative species such as $O_2^{\cdot -}$ and H_2O_2 by neutrophils and monocytes in response to bacteria or fungi infection [131]. Myeloperoxidase (MPO), abundantly expressed in neutrophils, is an enzyme that participates in innate immune response due to its ability to generate strong chlorinating oxidants such as hypochlorous acid from H_2O_2 produced by neutrophils [132]. Furthermore, it oxidizes tyrosine to tyrosyl radical using H_2O_2 as an oxidizing agent [131]. Hermann and colleagues demonstrated that SA acts as a catalyst of lipid oxidation in LDL in the presence of MPO due to its ability to form phenoxyl radicals [133]. Conversely, the main metabolite of SA, gentisic acid, not only inhibited the MPO system but also counteracted the tyrosyl radical-promoted LDL oxidation [133] (Fig. **4**). It is noteworthy that gentisic acid has the ability to overcome the salicylate-stimulated LDL oxidation at salicylate/gentisic acid ratios found in plasma of patients under ASA treatment [133]. Thus, it seems that the overall beneficial effect of ASA therapy on the prevention of cardiovascular risk might be partially mediated by the antioxidant effect of gentisic acid on LDL lipid oxidation caused by phenoxyl radicals [133].

8. OTHER PROMISING PHARMACOLOGIC MECHANISMS

The clinical use of ASA in inflammatory diseases requires doses exceeding those usually needed to inhibit COX (1-2mM) [14]. Moreover, SA, even in high doses, is a weak inhibitor of COX but an effective anti-inflammatory agent which suggests that at least at high doses, ASA and SA effects are COX-independent [6]. Furthermore, ASA and SA have been shown to block NF-κB-mediated gene expression at suprapharmacological concentrations [40] (Fig. **4**). Also, these molecules inhibit COX-2 and iNOS protein expression through blocking the binding of CAAT/enhancer binding protein β (C/EBPβ) [134-136] (Fig. **4**). More recently, a possible mechanism by which both ASA and SA may reduce vascular oxidative stress has been suggested. These salicylates inhibited the expression of LOX-1 that was induced by oxidized low-density lipoprotein (oxLDL) in endothelial cells [15]. These recent studies provide new insight into novel pharmacological actions of ASA and SA, which implicate the transcription factors C/EBPβ and NF-κB, as well as the receptor LOX-1, as potential targets for therapy of inflammation and oxidative stress-mediated tissue injury.

8.1. Inhibition of NF-κB, AP-1 and Protein Kinases

Nuclear factor kappa B is a pivotal transcription factor involved in the innate immune response, inflammation, apoptosis and stress responses. The dimeric Rel/NF-κB family of transcriptional factors regulates the expression of proinflammatory genes encoding various cytokines (e.g., IL-1, IL-2), chemokines (e.g., IL-8, monocyte chemotactic protein 1), enzymes (e.g., iNOS, COX-2), adhesion molecules (e.g., ICAM-1, VCAM-1) and immunoreceptors that mediate the innate immune response [137] (Fig. **4**). The mammalian NF-κB transcription factor family consists of five cellular proteins:

Rel (c-Rel), RelA (p65), RelB, NF-κB1 (p50 and its precursor p105) and NF-κB2 (p52 and its precursor p100) [138]. NF-κB exists as homo- or heterodimers and are retained inactive in the cytoplasm of most cells, bound to the inhibitory proteins, IκBs [139]. Nuclear factor kappa B is activated in response to various stimuli such as TNF-α, IL-1 and pathogen associated molecular patterns which, through different receptors, including tumor necrosis factor receptor (TNFR) and Toll-like receptor (TLR)-interleukin-1 (IL-1) receptor (IL-1R) superfamily, activates the IκB kinase complex (IKK) [140]. This complex is composed by a heterodimer of two catalytic subunits, IKKα and IKKβ that are linked to a regulatory subunit, IKKγ or NEMO [141]. There are two distinct pathways that lead to NF-κB activation and thereby its translocation from the cytoplasm to the nucleus [140]. The classical or *canonical* signaling pathway begins with the activation of IKKβ *via* an IKKγ-dependent manner that catalyzes the phosphorylation of IκBs, polyubiquitination and proteasome-mediated degradation [140]. The released dimer, p50-p65, binds to DNA and activates gene transcription. The new signaling pathway, known as the *non canonical* pathway is independent of IKKγ and is strictly dependent on NF-κB inducing kinase (NIK) phosphorylation that activates IKKα homodimers with the consequent nuclear translocation of p52-RelB dimer [140].

Kopp and Ghosh were the first to describe that SA and ASA impair the activation of NF-κB. In LPS-stimulated cells, ASA and SA blocked the LPS-induced phosphorylation and proteolysis of IκB, which suggests that the inhibition of NF-κB was mediated through the inactivation of the classical signaling pathway [40]. Noteworthy, the concentrations used of 2-20 mM for ASA and 2-5 mM for SA, were near the range of the plasmatic concentrations (1-2mM) attained during anti-inflammatory therapy [39-40]. Even though concentrations of 10-20 mM might be considered suprapharmacological, such high concentrations could be found in patient´s plasma under treatment of chronic inflammatory diseases such as arthritis [142]. Succeeding studies confirmed that both ASA and SA inhibit NF-κB activation by avoiding phosphorylation and subsequent dissociation of IκB [143]. The mechanism underlying the inhibition of the IKK complex activity *in vivo* and *in vitro* is due to the ability of salicylates to compete with ATP and directly binding to the subunit IKKβ, therefore preventing the degradation of IκB [144]. In addition, salicylates are able to suppress downstream targets of NF-κB as shown by the decrease of the TNF-α-induced mRNA levels of the adhesion molecules VCAM-1 and ICAM-1 [143].

Our research group has been exploring the NF-κB inhibitory effect of salicylates as an antidote for paraquat poisonings. It was clearly shown that SA (200 mg/Kg i.p.) and lysine ASA (100 mg/Kg i.p.) led to full survival of paraquat intoxicated rats [145-147] [148]. Although several other mechanisms were also proposed for the protective effect of salicylates, the dose range used inhibited PQ-induced NF-kB activation [146]. The finding that NF-κB activity is inhibited by salicylates indicates that their anti-inflammatory activity is partially related to the inhibition of this transcription factor. However, this hypothesis has been questioned since other studies have shown that SA, at concentrations that cause maximal inhibition of COX-2, has no effect on NF-κB activation [149]. Saunders and colleagues [134] also corroborated these results and additionally proposed that the reduction of COX-2 and iNOS expression is rather mediated by inhibition of C/EBP β binding and transactivation [136] (Fig. **3**). SA, at therapeutic concentrations, exerts direct inhibitory action on p90 ribosomal S6 kinase (RSK) thus reducing RSK-induced C/EBPβ binding and transcriptional activation of COX-2 [135]. The inhibition of the RSK family of kinases results in a inactivation of cAMP response element binding protein (CREB) and IκBα, which leads to an inhibition of CREB and NF-κB-dependent gene transcription [150]. This pleiotropic transactivator is involved in the expression of various pro-inflammatory cytokines and mediators and therefore the anti-inflammatory and antioxidant properties of salicylates might be partially attributable to their ability to prevent C/EBP β activation [151]. Thus, ASA and SA at pharmacological concentrations (10 μM-1 mM) selectively inhibit IL-1β and phorbol ester myristate induced-C/EBPβ binding to DNA specific sites whereas at suprapharmacological concentrations (>5mM) inhibit TNF-α-induced-NF-κB activation [152] (Fig. **4**).

Among reactive oxygen species, $HO^{\cdot}$ has been reported as the main messenger for NF-κB activation (Shi *et al*. 1999b). Thus, it seems likely that the $HO^{\cdot}$ scavenging activity of salicylates may also be involved in the inhibition of this transcriptional factor (Shi *et al*. 1999a). However, additional studies are necessary to support this assumption.

Besides controlling the transcription factors NF-κB, CREB and C/EBPβ, salicylates also target another transcription factor, the activator protein 1 (AP-1) [153]. AP-1 is a dimeric protein complex (homodimeric or heterodimeric) comprising the products of the *jun* and *fos* oncogene families [154]. The activation of AP-1 is mediated by a number of diverse signals that include UV radiation, oxidative stress, TNF-α, interferon γ and antigen binding by T or B lymphocytes [155]. Some of the genes known to be regulated by AP-1 are involved in the immune and inflammatory responses, tumor promotion and progression, and most of these genes overlap with the NF-κB target genes [156]. AP-1 is induced and regulated by the same stimuli as NF-κB and is likewise inactivated by salicylates (Dong *et al*. 1997) and probably through its free radical scavenging properties. The inhibition of AP-1 activation was also recently corroborated by our group, also representing a potential mechanism that ultimately contributed for the beneficial protective effects of salicylates in paraquat intoxicated rats [146-147].

8.2. Inhibiton of Lectin-Like Oxidized LDL Receptor-1?

One of the major causes of the coronary artery diseases is atherosclerosis and the consequent rupture of the atherosclerotic plaque formed in the vessels [157]. Atheroma plaques are constantly stressed by a variety of biomechanical and hemodynamic forces, pro-oxidant activity and reduced NO synthesis, release of collagen degradating metalloproteinases (MMPs) and deposition of oxLDL, that may precipitate or trigger the rupture of vulnerable plaques [157].

Recently, Metha and colleagues [15] have proposed a new pathway by which ASA may protect endothelial cells from oxidative stress injury. Cultured coronary artery endothelial cells exposed to oxLDL show an increment of both (LOX-1) mRNA and protein levels. ASA decreased the expression of the oxLDL-induced-LOX-1 mRNA and protein in a time and dose-dependent manner (Fig. **4**). LOX-1 was first identified in endothelial cells but it is currently known that LOX-1 is expressed in macrophages, vascular smooth muscle cells and platelets [158]. LOX-1 is recognized as the molecule that induces endothelial dysfunction and apoptosis, mainly triggered by oxLDL but also by other stimuli such as shear stress, endothelin, angiotensin II and activated platelets [158]. Activation of LOX-1, as consequence of O_2^{-} generation, induces RhoA and Rac1*via* MT1-MMP, which result in NADPH oxidase activation [159]. LOX-1 reduces endothelial NO production through reaction with NADPH oxidase-generated O_2^{-} and by LOX-1-mediated downregulation of eNOS [160]. Moreover, LOX-1 plays a role in the mediated NF-κB activation and expression of adhesion molecules and chemokines by oxLDL [161]. Additionally, stimulation of LOX-1 promotes apoptosis by increasing the pro-apoptotic factor Bax and reducing the anti-apoptotic factor Bcl2, and activates several kinases from the family of the mitogen-activated protein kinase (MAPK) signaling pathway [158, 162]. SA exerted a similar LOX-1 inhibitory effect but indomethacin, a cyclooxygenase inhibitor, failed to suppress LOX-1 expression [15]. This is not surprising since both ASA and SA but not indomethacin, inhibit O_2^{-} genera-

tion, which is a known regulator of LOX-1 expression [160]. The suppression of LOX-1 expression is correlated with downstream targets of this receptor, namely p38MAPK and matrix metalloproteinase-1 (MMP-1) (Fig. **4**). ASA prevented p38MAPK oxLDL-induced phosphorylation and consequently decreased MMP-1 activity and protein expression [15]. It seems that MMP-1 plays a role in both plaque vulnerability and in expansive arterial remodeling, leading to its activation and degradation of collagen within the atherosclerotic plaque, which can result in its rupture [157].

Furthermore, ASA prevents endothelium dysfunction during atherosclerosis [163] probably by protecting LDL against oxidative modification [164]. In hypercholesterolemic rats, ASA treatment significantly reduced lipid peroxidation and avoided reduction in glutathione content [13, 165]. The mechanism whereby ASA protects LDL from oxidation is most likely related to its antioxidant activities specifically its hydroxyl radical scavenging ability [77], inhibition of basal O_2^- production *via* vascular NADPH oxidases [11], chelation of free cytosolic iron [27], induction of ferritin synthesis [31] and by inhibiting free radicals generation through the prostaglandin synthase reaction [166] (Fig. **4**). Oxidation of native LDL results from lipid peroxidation products binding to lysine residues of apolipoprotein B and a mechanism by which ASA may inhibit at least the consequences of LDL-oxidation is acetylation of these residues [164].

9. CONCLUDING REMARKS

Nonsteroidal anti-inflammatory drugs have been used to treat arthritis since the beginning of the 19th century, when the analgesic and anti-inflammatory properties of SA were first discovered [167]. Despite of 110 years of clinical use, complete knowledge of ASA and its metabolites or other salicylates is still evolving with newly discovered pharmacological properties and clinical applications.

Both ASA and SA have remarkable hydroxyl scavenging properties, which explain the neuroprotection [168] and cytoprotection in models of ischemia/reperfusion injury [26]. High concentrations of salicylates interfere with several kinases, including the MAPK cascade and the transcription factors NF-κB and AP-1 responsible for the expression of a myriad of pro-inflammatory and pro-oxidant enzymes and cytokines [39, 156]. Several authors argue that the concentrations used to interfere with NF-κB and AP-1 and other cellular kinases are not tolerable as they cause severe adverse effects in humans. Nevertheless it was shown that at therapeutic concentrations, SA and ASA inhibit COX-2, iNOS and IL-4 gene expression through interfering with binding of C/EBPβ [134, 149]. Even though both salicylates have overlapping activities, ASA has shown unique biological functions namely the induction of ferritin and synthesis of 15-epi-lipoxin A_4 [31, 34, 50]. These novel lipid mediators could be considered as possible antioxidant effectors of ASA since it was demonstrated that ATL increases NO release, HO-1 expression, prevented nuclear NF-κB translocation and inhibited NAD(P)H oxidase activity, which was previously observed for ASA but not for SA or other NSAIDs [35-37].

Besides its own potent antioxidant activity, ASA yields two bioactive metabolites, SA and gentisic acid [79, 133], which may amplify the beneficial effects. Collectively, ASA has shown a broad of remarkable antioxidant mechanisms that are in accordance with the successful clinical use in the treatment and prevention of cardiovascular diseases and more recently as a chemopreventive drug against colorectal tumor.

ABBREVIATIONS

5-LO	=	lipoxygenase
15-epi-LXA_4	=	aspirin-triggered carbon-15 epimers lipoxins
15-HETE	=	15S-hydroxyeicosatetraenoic acid
AP-1	=	activator protein 1
ATL	=	acetylsalicylic acid-triggered lipoxins
ASA	=	acetylsalicylic acid
ATP	=	adenosine triphosphate
Bax	=	bcl-2–associated X protein
cGMP	=	cyclic guanosine monophosphate
CO	=	carbon monoxide
COX	=	cyclooxygenase
CREB	=	cyclic adenosine monophosphate response element-binding
C/EBP β	=	CAAT enhancer binding protein beta
DHBA	=	dihydroxybenzoic acid
eNOS	=	endothelial nitric oxide synthase
E-selectin	=	endothelial adhesion molecule 1
Fe(II)	=	ferrous ion
Fe(III)	=	ferric ion
GA	=	gentisic acid
H_2O_2	=	hydrogen peroxide
$HO^{\cdot}$	=	hydroxyl radical
HO-1	=	heme oxygenase 1
ICAM-1	=	inter-cellular adhesion molecule 1
IL	=	interleukin
iNOS	=	inducible nitric oxide synthase
IKK	=	IκB kinase complex
LDL	=	low density lipoprotein
LOX-1	=	lectin-like oxidized low-density lipoprotein receptor 1
LPS	=	lipopolysaccharide
LXA_4	=	lipoxin A4
MAPK	=	mitogen-activated protein kinase
MMP	=	matrix metalloproteinase
NAD(P)H	=	reduced nicotinamide adenine dinucleotide phosphate oxidase
NF-κB	=	nuclear factor kappa B
NO	=	nitric oxide
NSAID	=	non-steroidal anti-inflammatory drugs
O_2^-	=	superoxide radical
$ONOO^-$	=	peroxynitrite
oxLDL	=	oxidized low density lipoprotein
PMN	=	polymorphonuclear
RNS	=	reactive nitrogen species
ROS	=	reactive oxygen species
SA	=	salicylic acid
SAAG	=	salicyl acyl glucuronide
SAPG	=	salicyl phenolic glucuronide
SHRSP	=	stroke-prone spontaneously hypertensive rats
SOD	=	superoxide dismutase
SUA	=	salicyluric acid
TNF-α	=	tumor necrosis factor alpha
TXA_2	=	thromboxane A2
VCAM-1	=	vascular cell adhesion molecule 1

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Commentary I

Paraquat research: do recent advances in limiting its toxicity make its use safer?

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COMMENTARY

Paraquat research: do recent advances in limiting its toxicity make its use safer?

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Keywords
paraquat; human poisoning; Parkinson's disease; quinone oxidoreductase 2; survival

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The use of the herbicide paraquat (1,1′-dimethyl-4,4′-bipyridylium dichloride; PQ) has been fiercely challenged due to its severe acute toxicity, putative neurotoxicity after long-term exposure and lack of antidotes. Breakthrough research on PQ is therefore required for an effective risk control and to allow a safer use of PQ in the future. The silencing or inhibition of quinone oxidoreductase 2, a NAD(P)H-independent flavoenzyme, was shown to significantly attenuate PQ toxicity *in vitro*, in primary pneumocytes and astroglial U373 cells, and to strongly antagonize PQ-induced systemic toxicity and animal mortality. The novel results reported in this issue of *BJP*, added to recent findings using sodium salicylate and lysine acetylsalicylate, in which full survival of PQ-intoxicated rats was also achieved, open the door for new preventative and therapeutic strategies that may lead to safer use of this effective pesticide.

LINKED ARTICLE
This article is a commentary on Janda *et al.*, pp. 46–59 of this issue. To view this paper visit http://dx.doi.org/10.1111/j.1476-5381.2012.01870.x

Abbreviations
EU, European Union; PQ, paraquat; QR2, quinone oxidoreductase 2

Comment

In the present issue of the British Journal of Pharmacology, the research group supervised by Vincenzo Mollace reports a new finding of great interest concerning the antidotal effect against paraquat (PQ)-induced toxicity, attained through inhibition of quinone oxidoreductase 2 (QR2) (Janda *et al.*, 2012). This research may lead to safer paraquat use.

The introduction of PQ (1,1′-dimethyl-4,4′-bipyridylium dichloride) as an herbicide in 1962 has brought to the world one of the most controversial and studied pesticides over the last 50 years. At first, it appeared as a cheap and extremely effective, fast-acting and non-selective foliage-applied contact herbicide, killing a wide range of grasses and dicotyledonous weeds, and its use rapidly became widespread worldwide. However, it soon became notorious due to its toxic effects; human poisonings with this compound were frequently fatal due to its severe acute toxicity and lack of antidotes. For this reason, PQ was held responsible for thousands of deaths from both accidental and voluntary ingestion (the majority of the cases), as well as from dermal exposure (Dinis-Oliveira *et al.*, 2008). In July 2007, the European Union (EU) Court of First Instance annulled the Directive 2003/112 authorizing the use of PQ in the EU. Among other reasons, concerns about potential links between PQ and the Parkinson's disease and the requirement of human protection, which prohibits any exposure higher than the acceptable operator exposure level, as well as the protection of animal health, dictated the ban. In parallel, several non-governmental organizations were involved in fierce campaigns for a global ban of PQ, and some countries are restricting its use or applying a phase-out procedure.

With such a dark shadow over this herbicide, PQ research is becoming a complicated issue, both for scientists and for grant providers. Is it worth investigating or providing grants for the research on a compound that seems to be doomed?

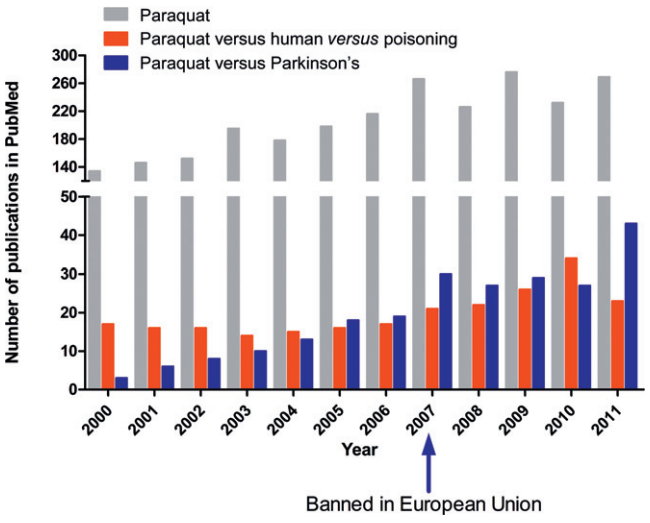

Figure 1

Number of publications related to paraquat per year, since 2000, in the National Library of Medicine database PubMed®.

Although some research groups, including our own, have already suffered this stigma in the financial support for PQ research, in our opinion, this area of research is more important than ever. It is noteworthy that PQ is still registered and applied in over 100 countries. The reasons for this are obvious and well beyond financial issues, namely its rapid action upon contact with the leaves, its lack of effect on roots and rhizomes, thus holding the soil together and preventing soil erosion, and its rapid deactivation by strong sorption to soil, thus limiting movement by leaching or surface run-off, decreasing possible ecotoxicological effects and allowing rapid reseeding or replanting after the killing of weeds (Bromilow, 2004). It is also worth mentioning that PQ can be applied safely when used according to the manufacturer's guidelines (Hart, 1987). PQ is highly hydrophilic and thus not absorbed through intact skin. Aerosolized PQ particles are large in diameter and thus do not reach the human alveoli when inhaled. Indeed, typical spray equipment generates droplet sizes with a median volume diameter over 100 μm (Dinis-Oliveira *et al.*, 2008).

Looking at the number of PQ publications per year in the National Library of Medicine database PubMed® (Figure 1), one rapidly realizes that, within this century, it doubled from 2000 (134) to 2007 (266) and then stabilized, but curiously the number of publications each year concerning PQ and human poisoning was fairly stable until 2007, after which it showed a sustained increase. Even more interesting is the number of publications per year relating PQ to Parkinson's disease. In 2000, this number was residual (3) and then steadily increased up to 10 times in 2007 (30), to stabilize thereafter, although another publication peak appears in 2011. These data suggest that fatalities due to PQ poisoning continue to be a heavy burden worldwide and that the possible involvement of PQ in the development or aggravation of Parkinson's disease is an area of increasing research interest.

It has long been postulated that NAD(P)H-dependent diaphorases are responsible for the *in vivo* reduction of PQ to the free radical monocation $PQ.^+$, which is then rapidly re-oxidized (returning to its original form) in the presence of O_2 with subsequent generation of superoxide anion radicals and other reactive oxygen species that are responsible for PQ toxicity (Dinis-Oliveira *et al.*, 2008). On the other hand, as now observed, the inhibition or silencing of the NAD(P)H-independent flavoenzyme QR2 significantly attenuates PQ toxicity *in vitro*, in primary pneumocytes and astroglial U373 cells, and strongly antagonizes PQ-induced systemic toxicity and animal mortality (Janda *et al.*, 2012). Most importantly, Wistar rats administered a specific QR2 inhibitor 2 h after PQ, which was subsequently maintained by several supporting doses, all survived an otherwise fatal dose of PQ (Janda *et al.*, 2012). The novel results reported in this issue of the BJP add to recent findings, which demonstrated that PQ-intoxicated rats all survived if treated with sodium salicylate or lysine acetylsalicylate (Dinis-Oliveira *et al.*, 2007, 2009), and open the door for new preventative and therapeutic strategies to be applied to PQ-intoxicated patients. Although this may constitute a *questione disputate*, breakthrough research on PQ, as in the present case, may ultimately lead to an effective risk control and allow the safer use of PQ in the future. Many other pesticides, such as organophosphates, are involved in a high number of voluntary intoxications, but do not pose a problematic treatment scenario and, therefore, continue to serve mankind. Hopefully, this may also happen with PQ, if further research significantly increases its safety.

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Review II

Pesticides exposure as etiological factors of Parkinson´s Disease and other neurodegenerative diseases – a mechanistic approach.

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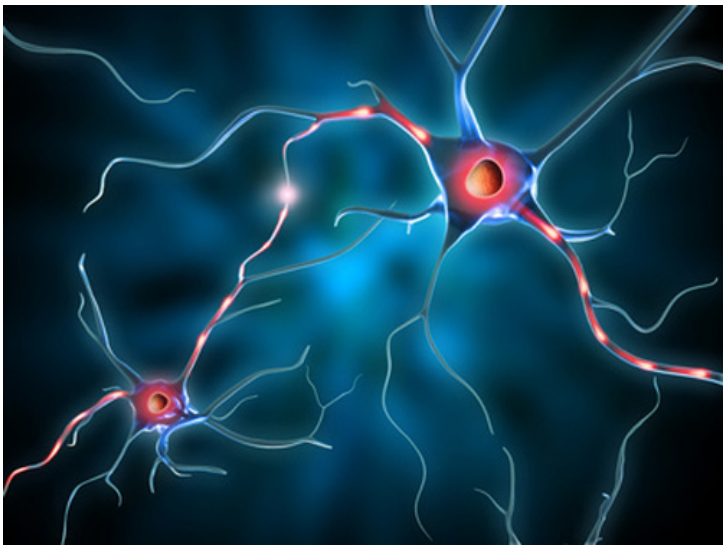

Pesticides exposure as etiological factors of Parkinson´s Disease and other neurodegenerative diseases - a mechanistic approach.

Running title: Pesticides and neurodegenerative diseases

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Abstract

The etiology of most neurodegenerative disorders is multifactorial and consists of an interaction between environmental factors and genetic predisposition. The role of pesticide exposure in neurodegenerative disease has long been suspected, but the specific causative agents and the mechanisms underlying are not fully understood. For the main neurodegenerative diseases such as, Parkinson's disease, Alzheimer's disease and Amyotrophic Lateral Sclerosis there are evidences linking their etiology with long-term/low-dose exposure to pesticides such as paraquat, maneb, dieldrin, pyrethroids and organophosphates. Most of these pesticides share common features, namely the ability to induce oxidative stress, mitochondrial dysfunction, α-synuclein fibrillization and neuronal cell loss. This review aims to clarify the role of pesticides as environmental risk factors in genesis of idiopathic PD and other neurological syndromes. For this purpose, the most relevant epidemiological and experimental data is highlighted in order to discuss the molecular mechanisms involved in neurodegeneration.

Keywords: Pesticides, Paraquat, Parkinson´s Disease, Alzheimer´s Disease, Amyotrophic Lateral Sclerosis, neurodegenerative diseases

1. Introduction

The World Health Organization currently estimates that around a billion people worldwide are affected by a neurodegenerative disease (WHO, 2006). As aging corresponds to the greatest risk factor for neurodegeneration, the prevalence of neurological disorders is expected to increase dramatically in next few years due to the higher life expectancy worldwide (Brown et al., 2005). Another risk factor for neurodegeneration, alongside with the aging process, is long-term/low-dose pesticide exposure. The role of pesticide exposure in the genesis of neurodegenerative diseases has been especially scrutinized for Parkinson´s disease (PD) (Franco et al., 2010).

PD is a progressive movement disorder characterized by progressive bradykinesia (slowness of voluntary movement), rigidity, rest tremor, and postural disturbances. Nonetheless, it is also increasingly recognized that non-motor symptoms, including autonomic and cognitive impairment, sleep disturbances, olfactory dysfunction, and depression occur in PD patients, and these features are probably due to the spread of the pathology beyond the basal ganglia (Shulman et al., 2011). The PD´s motor manifestations are attributed to the progressive loss of dopaminergic neurons in the *substantia nigra pars compacta* (SNpc) resulting in secondary dysfunction of the basal ganglia, which are involved in the initiation and execution of movements (Shulman et al., 2011). The subcellular hallmarks of PD are the intraneuronal inclusions of various structures consisting mostly of fibrillar α-synuclein (Lewy bodies and Lewy neurites) (Halliday et al., 2011).

The etiology of PD is currently unknown but it is assumed that there is a significant non-genetic contribution. It seems that the disease results from combination and accumulation of environmental exposures, and complex gene-environment interactions sustained by the slow and progressive development during aging (Cannon and Greenamyre, 2011; Dinis-Oliveira et al., 2006). Most of the forms of PD are idiopathic, but approximately 10-30% of the cases have a familial history, and in a minority of them the disease follows a Mendelian inheritance pattern. The disease is characterized by an early-onset (typically under 40 years) and so far 15 PD loci (PARK1-15) and 11 genes for PARK loci, specially *a-synuclein, leucine-rich repeat kinase 2, parkin, PTEN-induced putative kinase 1, DJ-1*, and *ATP13A2* have been described to cause typical forms of inherited PD or parkinsonian syndromes (Coppede, 2012). Parkinsonism is often observed as one of the symptoms in other monogenic diseases, when mutations in non-PARK loci (*MAPT, SCA1, SCA2, spatacsin, POLG1*) occur. In sporadic PD, genetic polymorphisms in four loci (*SNCA, MAPT, GBA* and *LRRK2*) are considered strong risk factors (Coppede, 2012). It is consensual that both etiologies share the same pathological pathways. SNpc is highly sensitive to diverse genetic, cellular and environmental factors that independently or concomitantly cause cell death over time. For instance, evidence suggests that mitochondrial dysfunction, accumulation of misfolded and aggregated proteins (ubiquitin-proteasome system and autophagy pathway impairment) and oxidative and nitrosative stress play an essential role in the pathogenesis of both idiopathic and familial forms of PD (Kanthasamy et al., 2012).

Since the discovery of the ability of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) to reproduce some of the features of PD, research has been focused on finding other environmental risk factors implicated in the etiology of PD, which revealed that occupational exposures to paraquat (PQ), maneb (MB) and rotenone have been associated with higher incidence of PD.

Besides PD, several studies have also suggested that Alzheimer´s disease (AD), amyotrophic lateral sclerosis (ALS), dementia and deficits in cognitive function are linked to occupational pesticide exposure (Cannon and Greenamyre, 2011; Chang and Wu, 2009; Kamel and Hoppin, 2004b; Kamel et al., 2012; Migliore and Coppede, 2009a; Sutedja et al., 2009). AD is a chronic disease characterized by progressive loss of memory and cognitive capacity, ultimately leading to dysfunction in daily life or work abilities. The neurodegeneration seen in AD involves two main protein aggregates, senile/amyloid plaques and neurofibrillary tangles (Palop and Mucke, 2010). Senile plaques are deposits of fibrils of the β-amyloid peptide, a fragment derived from the proteolytic processing of the amyloid precursor protein whereas neurofibrillary tangles are a compact filamentous network of helical filaments from hyperphosphorylated tau protein (Maccioni et al., 2001). Initially, the entorhinal cortex and hippocampus are particularly affected, as shown by the impaired synaptic transmission, especially reduction in the glutamatergic synaptic transmission strength and plasticity, and cholinergic dysfunction (Danysz and Parsons, 2012; Nyakas et al., 2011). The

cause for development of AD as other neurodegenerative diseases is not fully understood, however roughly 0.1% of the cases arise from mutation in 3 genes (*APP*, *PSEN 1* and *PSEN 2*) that result in a familial early-onset (<65 years) autosomal dominant forms (Migliore and Coppede, 2009b). Metals, pesticides, solvents, electromagnetic fields, brain injuries, inflammation, educational levels, lifestyles and dietary factors have been proposed as environmental AD risk factors (Cannon and Greenamyre, 2011; Dosunmu et al., 2007). Carbamates, organophosphates (OPs) and organochlorines are the pesticides more frequently associated with occupational exposure and AD (Hayden et al., 2010; Parron et al., 2011; Tyas et al., 2001; Zaganas et al., 2013).

Amyotropic Lateral Sclerosis (ALS) is a neurodegenerative disease evidenced by progressive loss of motor neurons at the anterior horn of the spinal cord and brain, resulting in progressive weakness, muscle atrophy, and respiratory failure within 3-5 years after diagnosis (Al-Chalabi et al., 2012). Around 5% of the cases are familial forms of ALS that arise from mutations in several genes including *SOD1, TARDBP, FUS, UBQLN2, C9orf72,* and *TAF15* (Al-Chalabi et al., 2012; Lill et al., 2011; Maccioni et al., 2001). Studies in mutations in *SOD1* have provided new insight into the pathogenesis of ALS, namely the generation of reactive oxygen (ROS) and nitrogen species (RNS), dramatic gliosis characterized by abnormalities of astrocytes, widespread astrocytosis, increased expression of inducible nitric oxide synthase (NOS) and activated microglial cells (Almer et al., 1999; Cha et al., 2000; Nagy et al., 1994). Interestingly, the neuronal cytoplasmic inclusions of ALS are constituted by aggregates of proteins encoded by the mutated genes described above (Ince et al., 2011). ALS and sporadic frontotemporal lobar degeneration share the same pathologic lesion, the 'Ubiquitin-only inclusion' body, within lower motor neurons and cerebral neurons (hippocampal and frontotemporal neocortex neurons), and both are considered proteinopathies (Ince et al., 2011). The remaining 95% of cases do not have an obvious family history of ALS and appear to occur sporadically throughout the community (Schymick et al., 2007). Despite this fact, the etiology of the majority of sporadic ALS cases is presumably due to several interactions between genetic and environmental factors. The genes that have been identified as being causative of ALS are related to DNA repair (*APEX1* and *hOGG1*), angiogenesis (ANG and VEGF), paraoxonases (*PON1*, *PON2* and *PON3*), iron metabolism (HFE), neurofilaments (*NEFH*), and survival motor neuron (*SMN1* and *SMN2)* (Maccioni et al., 2001; Migliore and Coppede, 2009b; Schymick et al., 2007). Several studies suggest that occupational exposure to pesticides is a significant risk factor of ALS (Bonvicini et al., 2010; Govoni et al., 2005; Kamel et al., 2005; Kanavouras et al., 2011; Qureshi et al., 2006). For instance, Horner and colleagues reported a two-fold increase in the risk of ALS among veterans of the 1991 Gulf War over the subsequent 10 years (Coffman et al., 2005; Horner et al., 2003; Miranda et al., 2008). Although the information about the chemicals to which soldiers were exposed is scarce and biased, the possibilities include OPs, other pesticides,

nerve gases, pyridostigmine, petrochemicals and depleted uranium (Spencer et al., 1998).

Recently, two different research groups conducted a retrospective meta-analysis of studies relating ALS and pesticides as a group (Malek et al., 2012), and one of them investigated the association of ALS with specific pesticides, using data from the Agricultural Health Study, a cohort including 84.739 private pesticide applicators and spouses (Kamel et al., 2012). From the eight case-control studies (Bonvicini et al., 2010; Chancellor et al., 1993; Deapen and Henderson, 1986; Granieri et al., 1988; Gunnarsson, 1994; McGuire et al., 1997; Morahan and Pamphlett, 2006; Savettieri et al., 1991) and one cohort study (Weisskopf et al., 2009), the major finding was the strong association observed between agricultural activities and ALS, although the chemical or class of agrochemical was not specified by the majority of studies (Kamel et al., 2012; Malek et al., 2012; Sutedja et al., 2009). On the other hand, in the Agricultural Health Study, ALS was not associated with pesticides as a group, but was associated with use of organochlorine insecticides, herbicides, pyrethroids, and fumigants (Kamel et al., 2012).

There is mounting evidence that long-term/low dose pesticide exposure is potentially neurotoxic and increases risk of PD and with lesser extent other neurological diseases, such as AD and ALS. PQ and MB are the most studied pesticides, though pesticides such as dieldrin, pyrethroids and OPs are also described as neurotoxics. The pesticides addressed in this review represent the five important classes of pesticides that have been more extensively studied in this matter. The neonicotinoids, acetamiprid and imidacloprid belong to a new class of insecticides and the latter is considered the most widely used within this class in the world. Nonetheless, so far neonicotinoids are considered safer than other compounds due to their higher selectivity for the nicotinic receptors of insects than mammalian receptors (Casida and Durkin, 2013). There are few studies reporting nervous system depression following acute poisoning with imidacloprid or acetamiprid, but currently there is no evidence or human data reporting health effects on humans after prolonged exposure to neonicotinoids (Imamura et al., 2010; Karatas, 2009; Phua et al., 2009; Proenca et al., 2005).

This review aims to clarify some of the mechanisms involved in the genesis of idiopathic PD and other neurological syndromes and the role of pesticides as environmental risk factors.

2. Paraquat

2.1 Oxidative stress and inflammation

The herbicide PQ has increasingly been reported in epidemiological studies to enhance the risk of developing PD. PQ belongs to the chemical class of bipyridyl (also called bipyridylium) quaternary ammonium herbicides characterized by two covalently linked methylpyridine rings (Calderbank, 1968). The toxicity of PQ has been extensively described concerning the effects to the main target organ, the lungs, and also the kidneys, liver and heart (Dinis-Oliveira et al., 2008). However, only in the past decade the research has been focusing on the effect of PQ in the brain after several reports of brain damage in individuals exposed to lethal doses of PQ (Baltazar et al.,

2013; Dinis-Oliveira et al., 2006; Grant et al., 1980; Hughes, 1988; Soontornniyomkij and Bunyaratvej, 1992). The mechanisms of PQ-induced neurotoxicity are not fully comprehended yet, but several pathways have been proposed: induction of oxidative stress, mitochondrial dysfunction, apoptosis and autophagy, inhibition of the ubiquitin-proteasome system, induction of synucleinopathy and tauopathy (Dinis-Oliveira et al., 2009; Franco et al., 2010) (Fig. 1).

A reliable amount of evidence has demonstrated that oxidative stress is considered to have a key role in the pathogenesis of PD (Drechsel and Patel, 2008). Dopaminergic neurons are markedly exposed to oxidative stress injury, mainly due to the oxidative metabolism of dopamine (DA), which contributes to sustain a higher production of ROS in the SNpc when compared to other regions of the brain (Fahn and Cohen, 1992). The other factors contributing to the selective vulnerability of the nigrostriatal system to oxidative stress include the following:

(a) The capability of DA auto-oxidation products, quinones and semiquinones to adduct proteins containing a thiol group such as glutathione and DNA (Graham, 1978; Hastings, 1995; Levay and Bodell, 1993)

(b) High aerobic respiration activity (Bueler, 2009)

(c) Increased iron concentration in the *substantia nigra* that leads to the production of hydroxyl radical ($HO^{\cdot}$) through Fenton reaction (Andersen, 2004; Kaur and Andersen, 2004).

PQ is widely known as a redox cycling agent, capable of accepting one electron from several cellular diaphorases (enzymes that transfer one electron from NAD(P)H), mainly NADPH-cytochrome P450 reductase, to form the monocation radical ($PQ^{\cdot+}$). Other enzymes are able to reduce PQ^{2+} to $PQ^{\cdot+}$, which comprise mitochondrial NADH:ubiquinone oxidoreductase (Gray et al., 2007), xanthine oxidase, NOS (Day et al., 1999), NADPH-oxidases from Nox family (Cristovao et al., 2009), and thioredoxin reductase (Gray et al., 2007). The $PQ^{\cdot+}$ is then rapidly reoxidized, regenerating its parent compound, in the presence of O_2, originating superoxide ($O_2^{\cdot-}$). The $O_2^{\cdot-}$ produced can react with nitric oxide (NO) to form peroxynitrite ($ONOO^-$) or be dismutated by superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2). In the presence of iron (II), H_2O_2 is reduced to $HO^{\cdot}$, a far more damaging ROS that rapidly oxidizes DNA, proteins and lipids (Klein and Ackerman, 2003) (Fig. 1).

The role of oxidative stress in PQ-induced neurodegeneration has been demonstrated in several cellular models (Castello et al., 2007; Cocheme and Murphy, 2008; Shimizu et al., 2003b; Wu et al., 2005) and *in vivo* studies (Czerniczyniec et al., 2011; McCormack et al., 2005; McCormack et al., 2002; Thiruchelvam et al., 2000b; Wills et al., 2012). PQ exposure was shown to cause depletion of GSH and increase GSSG levels in the *substantia nigra* of mice (Kang et al., 2009), augment malondialdehyde and protein carbonyls concentration, as well as DNA fragmentation (Yang and Tiffany-Castiglioni, 2005). PQ-induced increase in NADPH oxidase expression might result from initial ROS produced by the PQ redox cycle or mitochondria (Cristovao et al., 2009) (Fig. 1). However, continued production of ROS might be sustained by constant generation of NADPH oxidase-mediated $O_2^{\cdot-}$. When cells

were pretreated with apocynin, a putative NADPH oxidase inhibitor, PQ-induced ROS generation and dopaminergic cell death were significantly reduced. PQ has been shown to activate other signaling pathways such as protein kinase delta (PKC δ) or ERK1/2, which were reported as NADPH oxidase transcriptional activators (Miller et al., 2007). Exposure of microglial cells to PQ resulted in a rapid phosphorylation of ERK1/2 and phosphorylation of the cytosolic subunits of NADPH oxidase by PKC δ, resulting in increased ROS production and cell death (Miller et al., 2007).

Increasing evidences support that microglia, the resident macrophages in the brain, are a chronic source of inflammation and ROS responsible for progressive neuron damage (Surace and Block, 2012). Purisai and colleagues demonstrated that microglial activation is a priming event leading to PQ-induced dopaminergic cell degeneration (Purisai et al., 2007) (Fig. 1). Furthermore, a single-PQ exposure triggered an increase in the number of cells with immunohistochemical, morphological and biochemical characteristics of activated microglia, including induction of NADPH-oxidase, but failed to cause oxidative stress and neurodegeneration (Purisai et al., 2007). However, when PQ was repeatedly administered or challenged with other pro-inflammatory stimuli, such as lipopolysaccharide (LPS), the susceptibility of dopaminergic neurons to toxic injury was dramatically exacerbated (Mangano and Hayley, 2009; Purisai et al., 2007). The current knowledge supports the hypothesis that inflammatory insults may influence dopaminergic neuronal sensitivity to subsequent environmental xenobiotics by modulating the state of glial and immune factors, and these findings may be important for multifactorial neurodegenerative conditions, such as PD (Mangano and Hayley, 2009; Miller et al., 2007; Surace and Block, 2012).

Subchronic systemic injection of PQ (10 mg/kg) to C57BL/6 mice induced a specific dose and age-dependent neurodegeneration of the dopaminergic neurons in the SNpc populations, whereas GABA-ergic cells in the *substantia nigra pars reticulata* and cholinergic neurons in the hippocampus were not affected (McCormack et al., 2002). Noteworthy, the authors claimed that the discrepancy observed between neurodegeneration and lack of significant DA loss represents another important feature of PQ model and is probably a reflection of compensatory mechanisms by which neurons that survive damage are capable of restoring neurotransmitter tissue levels (Ossowska et al., 2006). Other authors also corroborated the same findings (Kuter et al., 2007; Ossowska et al., 2005), nonetheless the combined exposure to PQ and MB resulted in nigral DA loss associated with decreased tyrosine hydroxylase (TH) protein levels and nigral dopaminergic neurodegeneration (Li et al., 2005; Thiruchelvam et al., 2002).

McCormack and colleagues reported that ferritin transgenic mice are resistant to PQ-induced neuronal loss and lipid peroxidation, possibly by avoiding the generation of $HO^{\cdot}$ Via Fenton reaction (McCormack et al., 2005). In the same study, wild type C57BL/6 mice showed simultaneous dose-dependent loss of nigrostriatal dopaminergic neurons and an increase in the counts of neurons

immunoreactive for 4-hydroxinonenal, and nitrotyrosine, biomarkers of lipid peroxidation and nitrosative damage, respectively. Similarly to the *in vivo* studies (McCormack et al., 2005; McCormack et al., 2002), midbrain cells exposed to a single treatment with 10 mM PQ did not show a decrease in the number of dopaminergic neurons, although sequential treatments with 10 mM PQ for 2 days considerably killed dopaminergic neurons (Shimizu et al., 2003b). These results strongly support that the constant exposure to low levels of PQ would lead to the vulnerability of dopaminergic neurons in the nigrostriatal system. Moreover, an indirect excitotoxic pathway involving the NMDA receptors has been proposed by Shimizu and colleagues (Shimizu et al., 2003a). Glutamate and ROS including NO have been hypothesized to play a pivotal role in neuronal cell loss (Sawada et al., 1996). The toxic mechanism of PQ involves the stimulation of glutamate efflux from non-NMDA receptors, resulting in activation of NMDA receptor-channels. The high intracellular influx of Ca^{2+} stimulates NOS. Released NO can diffuse to the dopaminergic terminals and further induce mitochondrial dysfunction and interaction with other ROS, with subsequent formation of peroxynitrite, resulting in continuous and long-lasting dopamine overflow (Shimizu et al., 2003a)

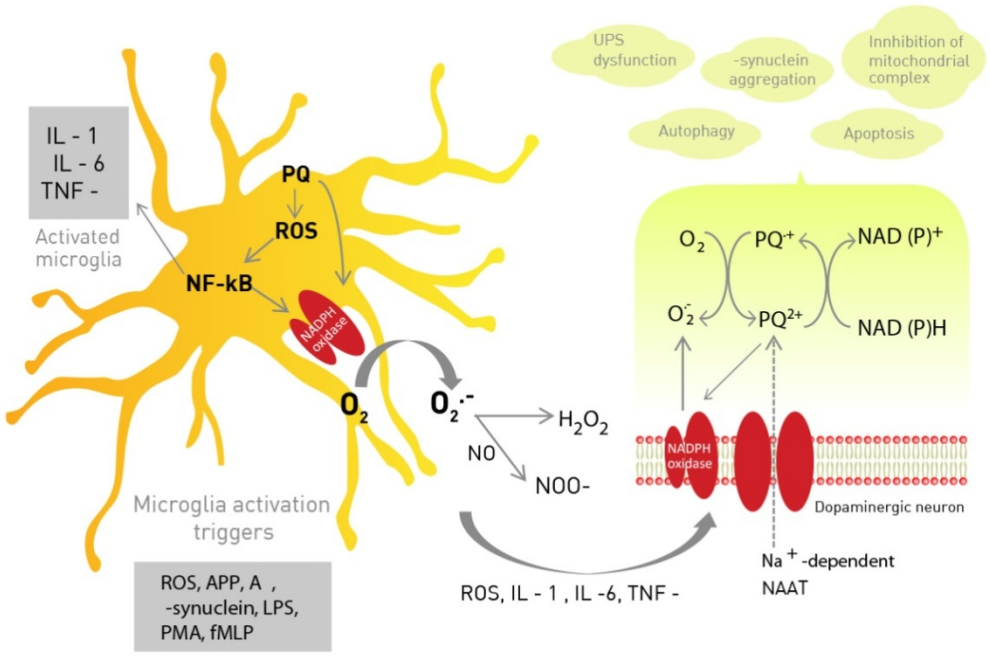

Figure 1. PQ increases NADPH oxidase expression possibly due to the ROS produced by the PQ redox cycle or mitochondria or the activation of NF-κB. Activated microglia releases inflammatory cytokines and superoxide radicals, which damage the adjacent neurons. Brain and striatal neurons uptake of PQ is supposed to be mediated by the neutral amino acid transport system (NAAT) in a Na^+-dependent manner. Within the cells, PQ^{2+} is reduced to $PQ^{\cdot+}$ by several oxidoreductases. The $PQ^{\cdot+}$ is then rapidly reoxidized, regenerating PQ^{2+}, in the presence of O_2, originating superoxide ($O_2^{\cdot-}$). The redox cycle leads to the generation of ROS and cellular damage. The other neurotoxicity mechanisms of PQ include mitochondrial dysfunction, apoptosis and autophagy, inhibition of the ubiquitin- proteasome system, induction of synucleinopathy.

2.2 Mitochondrial-derived ROS

Impairment of mitochondrial dynamics and function has emerged as one of the key mechanisms underlying the pathogenesis of both sporadic and familial PD (Bueler, 2009). Schapira and colleagues showed, for the first time, that the activity of the mitochondrial respiratory complex I (NADH-quinone oxidoreductase) is reduced in the *substantia nigra* of PD patients (Schapira et al., 1989). The susceptibility of nigral dopaminergic neurons to impairments of complex I activity is due to the oxidative metabolism of dopamine and iron content and, possibly from the low mass content of mitochondria in this region, compared to other neurons in the midbrain. The potential role of mitochondria in PQ-induced ROS production is controversial but ongoing research has revealed that mitochondria can be a major source of PQ-induced ROS production (Castello et al., 2007; Cocheme and Murphy, 2008). Mice deficient in two mitochondrial antioxidant enzymes, MnSOD and Gpx are more sensitive to PQ than wild type (Van Remmen et al., 2004). Mitochondrial expression of antioxidant enzymes, such as catalase or peroxiredoxin 5, protects against PQ toxicity more effectively than cytosolic expression (Mockett et al., 2003; Nguyen-nhu and Knoops, 2003). Castello and colleagues (Castello et al., 2007) claimed that mitochondrial respiratory complexes I and III both serve as targets for PQ-mediated ROS generation, with complex III showing a higher sensitivity, while other authors argued that complex I is the most likely site for damage (Cocheme and Murphy, 2008) (Fig. 2).

Mitochondrial aconitase (m-aconitase) is highly sensitive to $O_2^{\cdot -}$, which causes oxidation of the $[4Fe\text{-}4S]^{2+}$, promoting the removal of a labile iron that via Haber-Weiss reaction forms H_2O_2 (Cantu et al., 2009). In accordance, exposure to PQ was shown to induce m-aconitase-dependent increase in H_2O_2, Fe^{2+} and cell death as seen by the attenuation of H_2O_2 production when m-aconitase expression was reduced by RNA interference (Cantu et al., 2011).

2.3 Inhibition of proteossomal pathways and Synucleinopathies/ tauopathies

Progressive loss of DA neurons of the nigrostriatal system and deposition of filamentous α-synuclein aggregates are the main characteristics of PD (Puschmann et al., 2012). Indeed, besides the fact that α-synuclein is a natively unfolded protein that plays a central role in the control of synaptic membrane processes and biogenesis, when it becomes misfolded, it aggregates, and accumulates in neuronal inclusion bodies, the Lewy bodies (Bellucci et al., 2012). Remarkably, several studies indicate that multiplications or mutations of the *SNCA* gene are causative of autosomal dominant PD, and specific polymorphisms in the promoter region of the *SNCA* gene (REP1) increase the risk to develop PD. Other alterations that promote α-synuclein aggregation include nitration, hyperphosphorylation at Ser129, and the presence of DA adducts (Valente et al., 2012). PQ markedly induced the *in vitro* conformational changes in α-synuclein, and accelerated the rate of aggregation of α-synuclein (Uversky et al., 2002; Uversky et al., 2001). *In vivo* experiments have corroborated these results, showing that brain levels and aggregation of α-synuclein were significantly increased in PQ-treated mice, 2 days after

each of three weekly PQ injections and with protein levels returning to control values by day 7 post-treatment (Manning-Bog et al., 2002). α-Synuclein overexpression induces the formation of membrane pore-like structures that increase membrane conductance (Feng et al., 2010). The same authors concluded that leak channel conductance occurred prior to substantial cell death suggesting that pore formation may contribute to the overall cell vulnerability (Feng et al., 2010). More recently, Feng and colleagues demonstrated that co-treatment with PQ and DA in dopaminergic cells enhances α-synuclein-induced leak channel conductivity leading to a disruption of ionic imbalance, and eventually cell death (Feng and Maguire-Zeiss, 2011). PQ-treated mice striata showed significant accumulation of α-synuclein and hyperphosphorylation of Tau through activation of p-GSK-3β, a major Tau kinase (Wills et al., 2012). Notably, the specific sites of phosphorylation of Tau serine residues in PQ treated mice striata are the same sites found in PD *post mortem* striata (Wills et al., 2010). Besides, high levels of hyperphosphorylated (p-Tau) is strictly dependent on the presence of α-synuclein, as indicated by lack of any p-Tau formation in MPTP-treated α-synuclein −/− mice or in neuronal cells lacking α-synuclein (Duka et al., 2009; Duka et al., 2006). PQ, MPP^+, and rotenone, but not MB, are known to induce synucleinopathy and tauopathy (Duka et al., 2009; Duka et al., 2006; Hoglinger et al., 2005; Mitra et al., 2011; Wills et al., 2012).

Ubiquitin- proteasome system and autophagy are the two major pathways of degradation of misfolded, oxidized, and aggregated proteins. In PD, ubiquitin- proteasome system is impaired possibly due to depletion of ATP levels caused by mitochondrial dysfunction, oxidative stress or in cases of familial PD, mutations in *parkin* and *UCHL1* genes (Valente et al., 2012). Ubiquitin- proteasome system dysfunction leads to α-synuclein aggregation and the protein itself is capable of reducing even more the proteasomal activity (Branco et al., 2010). DJ-1deficient mice treated with PQ showed impaired proteasome activity and increased ubiquitinated protein levels. Nevertheless, the authors claimed that PQ exposure or deficiency in *DJ-1* gene alone did not stimulate a decrease in proteasome activity. The same study also showed that 19S ATPase Rpt6 and 20S β5 subunits and a transcription factor Nrf2 were decreased in *DJ-1*-deficient mice treated with PQ. On opposition, more recent studies have shown that administration of PQ (10 mg/kg), twice weekly for six weeks, significantly reduced the 26S proteolytic activity without loss of either 19S or 20S components or changes in the assembly of the 26S proteasome (Wills et al., 2012). The authors hypothesized that PQ seems to interact directly with the 20S component of the proteasome. In view of the fact that PQ induces overexpression α-synuclein and p-tau it is expected that proteasomal activity is reduced, due in part to the direct inhibitory effects of α-synuclein and p-Tau (Wills et al., 2012).

2.4 Cell death

PQ induces selective neurodegeneration in dopaminergic neurons in the SNpc triggering different mechanisms of cell death. Apoptosis induced by PQ has been shown to involve mainly the intrinsic mitochondrial pathway (Fei et al., 2008), and more recently, some

evidences suggest the contribution of endoplasmatic reticulum (ER) stress and (Niso-Santano et al., 2006) an autophagic process in neuronal cell death (Gonzalez-Polo et al., 2007b).

PQ triggers apoptosis *via* the intrinsic pathway by releasing cytochrome c and activation of caspase-9 due to the induction of Bcl-2 family members such as Bak, Bid, BNip3, and NOXA (Fei et al., 2008) (Fig. 2). Authors suggested that PQ neurotoxicity is mediated by a Bak-dependent mechanism by induction of Nox and BNip3 binding to Mcl-1 and Bcl-xL, respectively. This binding de-represses Bak, making it available to create mitochondrial outer membrane permeabilization with further release of cytochrome *c,* which will interact with Apaf-1 and procaspase-9 to create the apoptosome. The fully active apoptosome processes and activates executioner caspase-3 triggering apoptosis (Fei et al., 2008) (Fig. 2).

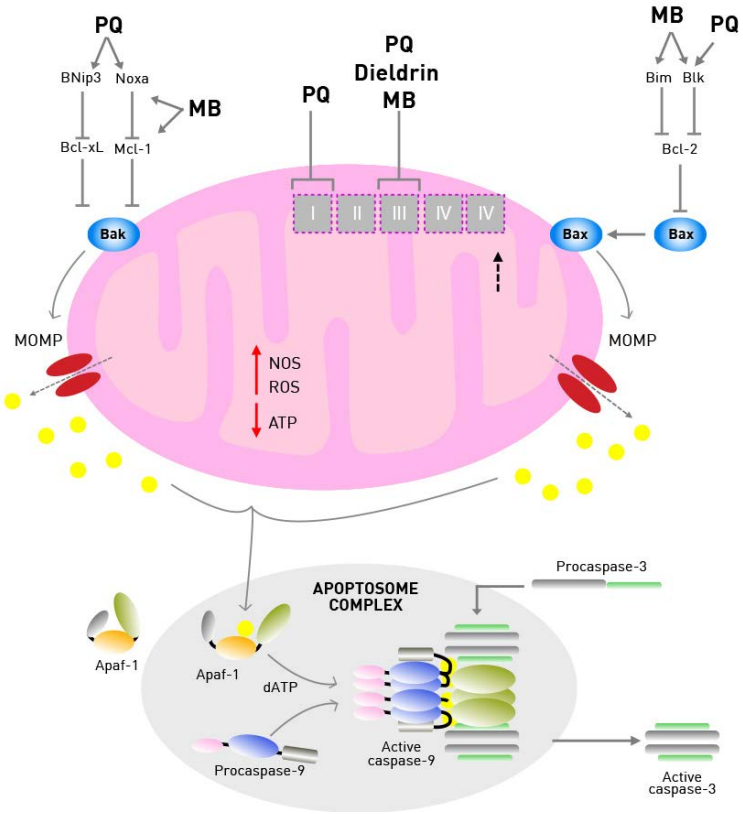

Figure 2. Mitochondrial dysfunction is a defect occurring early in the pathogenesis of both sporadic and familial Parkinson´s Disease (PD). Pesticide-induced dopamine neurons death has been also associated with their ability to alter the mitochondrial function. Paraquat (PQ) inhibits mainly the complex I and III, and Maneb (MB) and dieldrin inhibit only the complex III of the mitochondrial respiratory chain, generating reactive oxygen (ROS) and nitrogen species (RNS), leading to decreased ATP synthesis, oxidation of matrix proteins and mitochondrial DNA damage. PQ induces the BH3-only members Noxa and BNip3, and MB induces Noxa and Mcl-1. Noxa specifically binds to Mcl-1 and BNip3 binds to Bcl-xL, two major inhibitors of Bak. Binding of Noxa to Mcl-1 and BNip3 to Bcl-xL causes disinhibition of Bak. PQ and MB also disinhibit Bim, Blk, leading to disinhibition of Bax. The availability of Bak and Bax causes transient membrane disruptions, referred to as mitochondrial outer membrane permeabilization (MOMP), leading to the release of cytochrome c. Cytoplasmic cytochrome c complexes with Apaf-1 and procaspase-9 to form an apoptosome that activate executioner caspases, such as caspase-3, leading to apoptosis.

The ER is highly sensitive to oxidative stress, Ca^{2+} disturbances, and hypoxia (Boyce and Yuan, 2006). These disturbances cause accumulation of unfolded proteins in ER, triggering stress responses (Xu et al., 2005). Activation of inositol-requiring enzyme 1 (IRE1), apoptosis signal regulating kinase (ASK1), C/EBP homologous protein, and stressed-activated kinases lead to the activation/induction of pro-apoptotic Bcl-2 family members, which promotes the crosstalk between ER and the mitochondria-triggered apoptotic pathway, including release of cytochrome c from mitochondria and activation of caspase-3 (Paschen and Mengesdorf, 2005) (Fig. 3). An increasing body of evidence indicates that the stress-activated kinases, including c-Jun N-terminal kinase (JNK) and p38 kinase, play a critical role in the PQ-induced degeneration process (Choi et al., 2010; Klintworth et al., 2007). Sequential phosphorylation of JNK and the activation of caspase-3, and p53 transcription factors (Yang and Tiffany-Castiglioni, 2008) have been reported in animal and *in vitro* models of PD using PQ (Peng et al., 2004) (Fig. 3). These first studies demonstrated the involvement of p38/JNK signaling, however, how oxidative stress activates these pathways has not been established in culture or in animal models of PD. The current knowledge suggests that ASK1 acts upstream of JNK and p38 kinases throughout the phosphorylation of MKK3/6 and MKK4/7 (Yang et al., 2009) (Fig.3). Moreover, Niso-Santano and colleagues investigated the role of the transcription factor Nrf2, a master regulator of cytoprotective genes, and its target thioredoxin (Trx), which binds and inhibits ASK1. PQ induced a dose-dependent decrease in Trx levels correlated with a major increase in phosphorylated ASK1, suggesting that Nrf2/Trx is crucial in PQ-induced apoptosis (Niso-Santano et al., 2010) (Fig. 3).

Autophagy is a mechanism involved in the degradation of oxidatively damaged proteins and in organelle turnover. This phenomenon has been observed in neurons from patients with various neurodegenerative diseases such as Huntington's disease, AD and PD (Anglade et al., 1997; Kegel et al., 2000; Nixon et al., 2000). However, the implication of autophagy in PD and whether environmental xenobiotics upregulate or downregulate autophagy is still controversial (Janda et al., 2012). The accumulation of α-synuclein-rich protein inclusions induced by some pesticides (rotenone and PQ) suggests that the autophagy pathways are rather inhibited than promoted. The first *in vitro* studies reported that PQ triggers autophagy, shown by the significant increase in LC3II levels, weak inhibition of mTOR phosphorylation, and increase in LC3-GFP autophagic vesicles (Gonzalez-Polo et al., 2007a). The same authors claimed that PQ elicits autophagy as a defense mechanism to degrade the oxidized proteins by ROS since the inhibition of autophagy, using 3-MA, accelerated the apoptotic death process (Gonzalez-Polo et al., 2007a). From the six genes linked with hereditary PD, *α-synuclein*, *Parkin*, PTEN-induced kinase 1 (*PINK-1*), and *DJ-1* (Krebiehl et al., 2010) are the genes most strongly implicated in autophagy impairment. Gonzalez-Polo and colleagues defended that autophagy induced by PQ was dependent on *DJ-1*, as its knockdown reversed the autophagic response to PQ (Gonzalez-Polo et al., 2009). Unlike the *in vitro* experiments, the

only *in vivo* study suggests an impairment of macroautophagy and proteasome function upon exposure to PQ (Wills et al., 2012). Both MB and PQ increased the levels of mTOR, an inhibitor of autophagy, and reduced LC3 II to LC3 I ratio, despite increases in autophagic proteins, such as beclin 1 and Agt12. In parallel, increased mTOR was also observed in postmortem human PD striata, and a reduction in the LC3 II to LC3 I ratio as well (Wills et al., 2012). The controversial data raises doubts about the role of PQ in the autophagy pathway even though the *in vivo* studies suggest an impairment of macroautophagy. Further work will be necessary to elucidate the mechanisms underlying PQ-related modulation of autophagy.

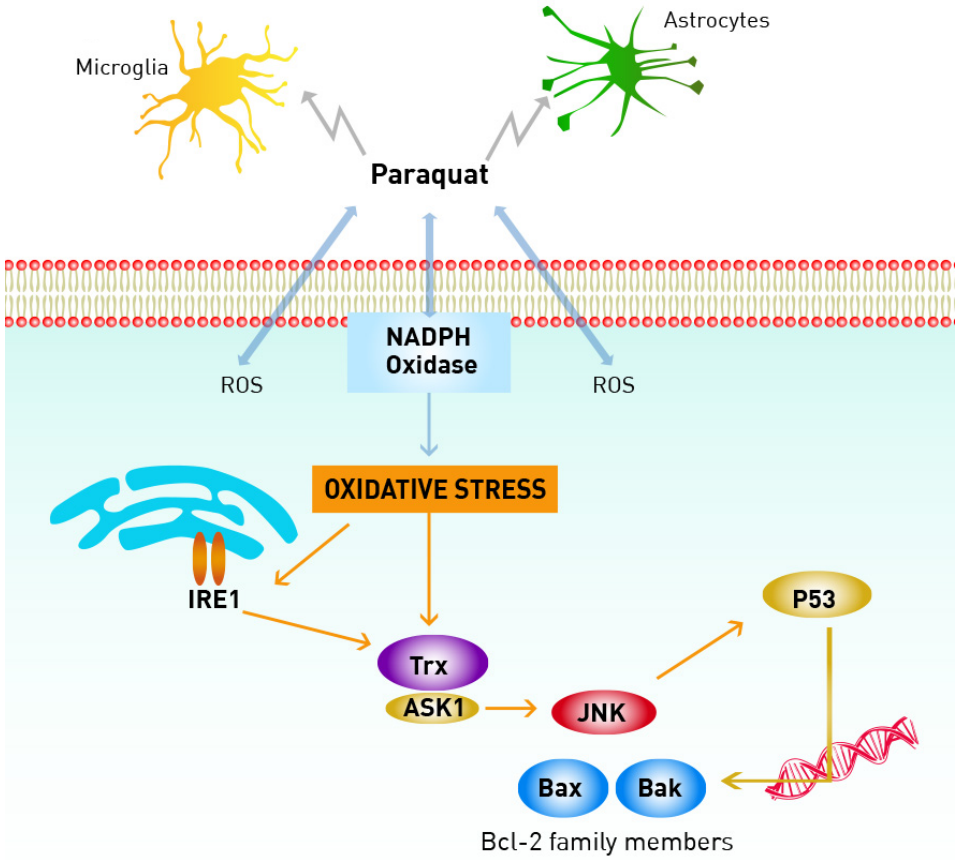

Figure 3. PQ production of ROS through the redox cycling, activation of microglia and astrocytes, inhibition of mitochondrial electron transport chain, and/or induction/activation of ROS generating enzymes such as NADPH oxidases, mediates the activation of cell death signaling cascades. Oxidative stress triggers the induction of endoplasmatic reticulum stress and activation of inositol-requiring enzyme 1 (IRE1), then activating the ASK1/JNK signaling cascade. PQ induces a dose-dependent decrease in Trx levels leading to an increase in phosphorylated ASK1, suggesting that Nrf2/Trx is crucial in PQ-induced apoptosis. Phosphorylation of JNK induces p53 transcription factor that lead to activation/induction of pro-apoptotic Bcl-2 family members, culminating in apoptosis. Adapted from (Franco et al., 2010).

2.5 Crosstalk between experimental and human data

Recently, case-control studies, cohort studies and cross-sectional studies were combined in two meta-analyses (van der Mark et al., 2012; Van Maele-Fabry et al., 2012). Despite not being completely consensual, the main conclusions reinforce the idea that there is evidence of an increased risk of PD associated with PQ exposure. The results suggested that heterogeneity was rather due to differences in the exposure assessment than with study design, source of control population, adjustment of results for potential confounders, or geographical area (van der Mark et al., 2012).

Kamel et al. (Kamel et al., 2007) analyzed the Agricultural Health Study (Alavanja et al., 1996) data from licensed private pesticide applicators and spouses to evaluate the relation of self-reported PD to pesticide exposure. There was a weak negatively association of prevalent PD with ever use of a pesticide and with personally mixing or applying pesticides and a positively association with incident PD. Incident PD was associated with the highest category of cumulative days of pesticide use at enrollment with personally applying pesticides more than half the time but not with prevalent PD. Considering only chemicals for which there were four or more exposed cases, OR´s for prevalent PD were elevated (>1.4). Actually, frequent use of PQ, the OR´s were 1.8 (95% CI 1.0, 3.4) for prevalent PD cases and 1.0 (95% CI 0.5, 1.9) for incident PD cases (Kamel et al., 2007).

Tanner and colleagues also analyzed the Agricultural Health Study data and conducted a case-control study focused to assess whether pesticides linked to mitochondrial dysfunction or oxidative stress, in a population with well characterized pesticide exposure, are associated with PD or clinical features of parkinsonism in humans (Tanner et al., 2011). From the eight pesticides classified as oxidative stressors, and from the seven classified as mitochondrial complex I inhibitors, only PQ and rotenone, respectively, were associated with PD. In 110 PD cases and 358 controls, use of PQ (OR = 2.5; 95% CI, 1.4–4.7 for men and women, and 2.7; 95% CI, 1.4-5.1 for men only), and rotenone (OR = 2.5; 95% CI, 1.3–4.7) were associated with PD, but for cumulative lifetime days of use, only PQ was positively correlated with duration of use (OR=3.6 95% CI, 1.6-8.1 for greater than the median). Despite the size of the study, wide variability of exposure, quality of diagnosis with movement disorders experts, and reliability of pesticide exposure information, the authors did not distinguish prevalent and incident cases, potential bias might have occurred during selection, and the subgroup analyses were not justified (Mandel et al., 2012). Also, the study could not rule out the possibility that the results were attributable to combined exposures or other agents other than those analyzed.

Even though long-term occupational exposure to pesticides might be linked with PD, most of the studies have not found a significant association with specific pesticides, namely, PQ. In a cohort study performed among men with high prevalence of parkinsonism and daily exposed to pesticides, Engel et al. found that the association of PD with PQ was negative (prevalence ratio= 0.8; 95% CI 0.5, 1.3). There was no correlation with duration of exposure as for the highest tertile of years of

exposure and for highest acre-years of exposure the prevalence ratios were < 1 (Engel et al., 2001). A study conducted in Taiwan with 120 patients and 240 controls, where the herbicide PQ is commonly sprayed over rice fields, reported an OR of 3.22 (95% CI, 2.41–4.31) for PD in PQ users compared with nonusers, and 6.44 (95% CI, 2.41-17.2 for the highest duration of use. However, subjects were highly exposed to other pesticides which difficult the comprehension of PQ involvement. (Hertzman et al., 1990) compared personal histories of 57 cases and 122 age-matched controls in British Columbia to identify possible environmental determinants of PD and reported an increased risk of PD for working in orchards (OR = 3.69, 95% CI = 1.34-10.27) and a marginally significant increased risk associated with working in planer mills (OR = 4.11, 95% Cl = 0.91-18.50). Based on Fisher's exact test of the association between PD development and PQ was statistically significant (p=0.01). In a population-based case–control study of incident PD in western Washington State, the only increased risk estimate was for men exposed to parathion, whereas for PQ the association was negative (OR=0.9, 95% CI 0.15- 5.43) (Firestone et al., 2010). These findings corroborate the previous study carried out by the same authors where there was no significantly increased odds ratio from exposure to PQ (OR=1.67; 95% CI 0.22-12.76) (Firestone et al., 2005). Three studies provided information about exposure to pesticides and increased risk of incident PD in agricultural areas of California (Costello et al., 2009; Gatto et al., 2009; Wang et al., 2011). In all studies, ambient exposure to pesticides was estimated from applications to agricultural crops employing a validated geographic information system (Gatto et al., 2009), concluded that, for PQ, the risk from well water consumption and ambient exposure were generally small and uninformative, which might be explained by the observations that exposure to PQ may require concomitant MB exposure to increase PD risk, as reported by Costello et al (see below). The latest study from the same group, reported that for combined exposure to ziram, MB and PQ, and for combined exposure to ziram and PQ there was a significantly increased odds ratio but exposure to PQ alone (OR = 1.26; 95% CI 0.86, 1.86) adjusted for age, sex, education, smoking, family history of PD and race was no significantly increase was observed (Wang et al., 2011).

Concerning the evidences for the role of PQ exposure (e.g. during application of this herbicide, production or following acute poisoning) in the etiology of PD, many aspects have to be addressed. Firstly, PQ is poorly absorbed through intact human skin (0.03 μg/cm^2 over 24 h), with only 0.3% of the applied dose being absorbed within 24 h (Wester et al., 1984). Furthermore, the few occupational studies performed have shown that even after a dermal exposure of PQ during application, urine levels were either undetectable (Chester et al., 1993; Van Wendel de Joode et al., 1996) or very low, with 83.3, 47.1 and 63.9% of the samples being below the LOQ before-, during- and after-paraquat spray days, respectively (Lee et al., 2009). PQ has low volatility and the fraction of respirable particles (<5 μm) produced by standard spray nozzles is low, limiting the absorption by inhalation. Assessment of PQ exposure during handling

of this herbicide reveals that dermal exposure is relatively high and that the degree of exposure via inhalation is below the permissible exposure limits set by United States National Institute of Occupational Safety and Health (Baharuddin et al., 2011). Moreover, other occupational studies do not report the quantification of PQ in biological samples and therefore understanding the extension of the PQ absorbed through skin and air is neglected (Dalvie et al., 1999;Machado-Neto et al., 1998).

Secondly, the question of how PQ, a charged hydrophilic compound, enters the brain remains to be clarified. In rodents, transport into brain has been proposed to occur via a specific neutral amino acid transporter, although it reaches a brain concentration ten times lower than in peripheral tissues (McCormack and Di Monte, 2003; Shimizu et al., 2001). Rappold and colleagues demonstrated that, when PQ^{2+} is reduced to the monovalent cation $PQ^{\cdot+}$ (in the presence of either a reducing agent or NADPH oxidase on microglia), it is efficiently taken up by cells through DAT and organic cation transporter 3 (Rappold et al., 2011). Other studies, in the rhesus monkey and C57BL/6J mice reported that PQ uptake and the pattern of PQ distribution in the brain is similar across species, with higher concentrations being found in areas of the brain not fully protected by the blood–brain barrier such as the olfactory bulb, pineal gland and lateral ventricles (Bartlett et al., 2009; Breckenridge et al., 2013). It was also reported that PQ is slowly eliminated from brain, but whether PQ is bound to tissues or organelles within the brain, or whether specific neurons or other cells selectively retain PQ is unknown (Breckenridge et al., 2013). On the other hand, *in vivo* evaluation of the toxicokinetics of PQ shows that it accumulates in a linearly way, with a half-life of approximately one month in adult C57BL/6J mice after a single dose (10 mg/kg) resulting in accumulation of similar levels of PQ in the different regions of brain (striatum, frontal cortex, hippocampus, and cerebellum) (Prasad et al., 2009; Prasad et al., 2007). Even when a low concentration of PQ (0.3 mg/ml) was given orally in drinking water, the brain concentration was ~0.12 ng/mg after 8 weeks of exposure (Prasad et al., 2009). However, neither of the studies quantified the urine and plasma levels, which hinder a possible correlation between the experimental data, the human exposure levels and epidemiological data.

Despite the literature evidences of the ability of PQ to reproduce some of the features of PD in mice models, the results should be interpreted carefully. Breckenridge and colleagues (Breckenridge et al., 2013) conducted a thorough experiment to evaluate the potential effects of PQ in the SNpc and striatum of male C57BL/6J mice. The scheme of treatment was similar to previous reported experiments (McCormack et al., 2005; McCormack et al., 2006; McCormack et al., 2002; Richardson et al., 2005) whereas PQ dose was 10 mg/kg i.p once per week for three consecutive weeks. Unlike others, their main finding is that under their conditions, there was minimal evidence of PQ-related neuronal degeneration without alteration of the concentration of dopamine (DA), homovanillic acid (HVA) or 3,4-dihydroxyphenylacetic acid (DOPAC), or increase DA turnover in the striatum. However, it should be noted that among the

published animal studies on PQ neurotoxicity there is a lack of consistency in dose, species or strain, age of animals and timing of treatment. The doses fluctuate between 5-25 mg/kg, the frequency of dose ranges from 1-2 times per week, the length of the study from 1-4 weeks. The majority of the studies with PQ and PD use as model the C57BL/6 or C57BL/6J mice but other studies have also use the swiss albino mice (Mitra et al., 2011) and the rats strain Sprague-Dawley or Wistar (Czerniczyniec et al., 2011; Songin et al., 2011). Breckenridge and colleagues (Breckenridge et al., 2013) tested one type of inbred mouse strain that is considered the most susceptible to PQ neurotoxicity, the C57BL/6J mice, at 2 months of age. The difference between studies might also be linked with the age of the animals. Peng and colleagues suggested that age contributes to the greater susceptibility to PQ due to the age-related iron accumulation in the *substantia nigra* (Peng et al., 2010; Peng et al., 2007; Peng et al., 2009). Other recent studies also report significant PQ-induced TH staining loss in SNpc in 4–6 month old C57BL/6J mice (Jiao et al., 2012; Yin et al., 2011).

3. MB and PQ + MB

In rodent models, MB was shown to alter behavioral function, reduce locomotor activity and increase aggressiveness (Morato et al., 1989). Direct injection of MB to the rat lateral ventricles resulted in selective dopaminergic neurodegeneration, induced extensive striatal DA efflux, and preferentially inhibited mitochondrial complex III (Zhang et al., 2003). Barlow and colleagues also reported that MB and other dithiocarbamates were able to increase synaptosomal DA accumulation *in vitro* at concentrations as low as 500 nM, possibly by delaying DA efflux (Barlow et al., 2003). Furthermore, MB was shown to increase the tissue content of $^{[14C]}$PQ *in vivo* by a mechanism that appeared to be distinct from the DA transporter (DAT), suggesting that dithiocarbamates might augment other xenobiotics neurotoxicity by modulating their toxicokinetics (Barlow et al., 2003).

MB has been shown to exacerbate the pro-oxidant condition through its ability to disrupt the glutathione antioxidant system in dopaminergic neurons (Barlow et al., 2005), to catalyze the auto-oxidation of DA (Fitsanakis et al., 2002), and to disturb the mitochondrial function, as an inhibitory uncoupler of the electron transport chain (Domico et al., 2006). Additionally, exposure of dopaminergic cells to 6 μM Mn-EBDC for 7 days produced not only significant neurotoxicity but also decreased proteasomal function, and led to α-synuclein aggregation with formation of cytoplasmic inclusions that were immunoreactive for α-synuclein (Zhou et al., 2004). Despite *in vitro* inhibition of proteasomal activity and induction of α-synuclein aggregation, MB effects *in vivo* are somewhat different. MB was ineffective in increasing α-synuclein or p-Tau levels. When PQ and MB were concomitantly administered, the effects were similar to when PQ was administered alone (increased α-synuclein aggregation and p-Tau levels), which suggests that MB does not enhance the effects of PQ (Wills et al., 2012). Moreover, unlike PQ, MB did not directly inhibit soluble proteasomal activity, nor did it intensify the direct inhibitory effect of PQ on this activity. In the same study, MB increased levels of the

autophagy inhibitor mammalian target of rapamycin, mTOR, suggesting impaired axonal autophagy, despite increases in certain autophagic proteins, such as beclin 1 and Agt12 (Wills et al., 2012).

Due to the fact that MB and PQ are used in geographically overlapping areas, and rural workers exposed to both pesticides have an increased risk of developing PD by 75% (Costello et al., 2009), several authors used PQ+MB as a PD model (Thiruchelvam et al., 2000a; Thiruchelvam et al., 2000b). C57BL/6 mice exposed to PQ (10 mg/kg) and MB (30 mg/kg), i.p., once a week for 4 weeks, showed reduced locomotor activity, significant DA fiber loss, and altered levels of DA and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (Thiruchelvam et al., 2000a). These effects were preferentially expressed in the nigrostriatal dopaminergic system (Thiruchelvam et al., 2000b). Fei and Ehtell, found that MB potentiates PQ neurotoxicity by triggering Bax-dependent cell death through activation of three strong Bak inhibitors, Bfl-1, Bcl-xL and Mcl-1, and also induced Bax activators that included Bik and Bim (Fei and Ethell, 2008).

Idiopathic PD is typically considered an aging-related neurodegenerative disorder, but its onset is still unclear, whether it could arise from events that occur during premature development, during adult life or from lifetime cumulative effects. Mice exposed developmentally to PQ+MB showed reduced levels of dopaminergic neurons (38% loss) in adulthood, while re-exposure of mice to the mixture of pesticides lead to a 70% loss of dopaminergic neurons in the *substantia nigra* and a concomitant decrease in locomotor activity (Thiruchelvam et al., 2002). Developmental exposure to PQ or MB alone produced minimal changes. Conversely, the response to a second stimulus in adult life was exaggerated, suggesting that there is a period of silent neurotoxicity that predisposes adult animals to the toxicity of a re-exposure. Moreover, subsequent studies have shown that ageing enhances sensitivity of nigrostriatal pathway to the combined exposure of PQ+MB (Thiruchelvam et al., 2003). Reduced levels of locomotor activity 24h and even 3 months after treatment were age-related, since 5 and 18 months old mice did not recover, whereas 6 week old mice exhibited total recovery. Also, levels of striatal DA and dopaminergic neurons in the *substantia nigra*, particularly for PQ+MB treatment in both 5 and 18 month old mice were decreased and unchanged 3 months after the final exposure (Thiruchelvam et al., 2003).

4. Dieldrin

Dieldrin, an organochlorine, is one of the most environmentally persistent insecticides. Despite having been banned in the 1970s in most of the developed countries, its low volatility, and high lipophilic properties lead to an extensively bioaccumulation and biomagnification in non-target species and soil. Nowadays, humans continue to be chronically exposed to dieldrin through contaminated food, polluted ground water, and environmental residues (Jorgenson, 2001).

Post mortem studies indicate that exposure to dieldrin is closely associated with PD, since dieldrin levels in the caudate nucleus from PD patients were significantly higher than those in

control brains (Corrigan et al., 1996; Corrigan et al., 1998; Corrigan et al., 2000). Also, previous studies revealed that dieldrin was detected in 6 of 20 PD brains, and in none of 14 control samples with a highly significant positive association between the insecticide and the diagnosis of PD (Fleming et al., 1994).

In vitro, dieldrin appears to be a relatively selective dopaminergic neurotoxin in mesencephalic cultures, indicated by the low neurotoxicity to GABA-ergic neurons compared to dopaminergic neurons (Sanchez-Ramos et al., 1998). Moreover, in rat and mice dopaminergic cell lines, dieldrin yields a depletion of intracellular DA levels, a decrease of DA metabolites, including 3,4-dihydroxyphenylacetic acid and homovanillic acid (Hatcher et al., 2007), a depolarization of mitochondrial membrane potential, generation of ROS (Chun et al., 2001), and apoptosis (Kitazawa et al., 2001) (Fig. 4). Dieldrin also activates brain microglia, inducing NADPH-dependent ROS production (Mao et al., 2007). A mechanism underlying dieldrin-induced apoptosis has been recently proposed. Kitazawa and co-workers reported that PC12 cells exposed to dieldrin release cytochrome c, which is followed by the activation of the caspases cascade and caspase-3-dependent proteolytic activation of PKC δ (Kanthasamy et al., 2008; Kitazawa et al., 2003) (Fig. 4). In accordance, dieldrin-induced Poly (ADP-ribose) polymerase cleavage, chromatin condensation and DNA fragmentation, and caspase-3 activation were completely blocked in Bcl-2-overexpressed PC12 cells as compared to control cells. These findings suggest that dieldrin primarily alters mitochondrial function to initiate apoptotic cell death, since overexpression of the anti-apoptotic protein Bcl-2 prevents these events (Kitazawa et al., 2004). In mesencephalic dopaminergic neurons, dieldrin can rapidly induce the hyperacetylation of histones, specifically histones H3 and H4. The histone hyperacetylation in the striatum and *substantia nigra* was also observed in mice exposed to dieldrin (5.0 mg/kg) for 30 days (Fig. 4). The authors also found that the protein level of CBP, a well-known histone acetyltransferase, was increased in a time-dependent manner. This fact might be due to dieldrin-induced proteasomal dysfunction, resulting in accumulation of pivotal histone acetyltransferase. The inhibition of CBP attenuated dieldrin-induced histone acetylation, caspase-3 activation, and PKC δ proteolytic activation, and DNA fragmentation in dopaminergic neurons (Song et al., 2010 287).

Dieldrin has been shown to induce a conformational change in α-synuclein and promote fibrillization of α-synuclein (Uversky et al., 2001) (Fig. 4). The overexpression of α-synuclein has been reported to inhibit proteasomal function (Snyder et al., 2003). For that reason, Sun and colleagues exposed α-synuclein overexpressing dopaminergic neurons to dieldrin (Sun et al., 2005). Their results showed that dieldrin impairs ubiquitin proteasome function additively with α-synuclein, and enhances the susceptibility of dopaminergic neurons to apoptotic cell death.

Together, the results suggest that combination of α-synuclein overexpression due to genetic mutations or exposure to environmental pesticides that also increase α-synuclein levels, are likely to contribute to the

overall vulnerability of dopaminergic neurons (Sun et al., 2005).

As mentioned above, exposure to pesticides during the perinatal period or early age may result in either permanent damage, progressive lesions of the nigrostriatal dopaminergic system or enhanced adult vulnerability to a future neurotoxicant challenges. Perinatal exposure of mice to low levels of dieldrin (0.3, 1, or 3 mg/kg every 3 days) resulted in a long-term enhancement of protein and mRNA levels of the DAT and vesicular monoamine transporter 2 (VMAT2) (Richardson et al., 2006). The increase DAT:VMAT ratio appears to be correlated with higher susceptibility of dopamine neurons to degeneration in PD (Miller et al., 1999). Indeed, when dieldrin-exposed mice were challenged with MPTP (2×10 mg/Kg s.c) at 12 week of age, the neurotoxicity was exacerbated as shown by the increase of α-synuclein levels and a greater reduction of striatal DA, which was associated with a greater DAT:VMAT2 ratio.

Although dieldrin shows many features of PD including the ability to induce mitochondrial dysfunction, oxidative stress and apoptosis, induction of α-synuclein aggregation, and DA depletion, it fails to provoke motor deficits and dopaminergic neuron loss. Other issues are the lack of more extensive studies that associate dieldrin exposure with PD, the high concentrations used in *in vitro* and *in vivo* studies and whether induction of oxidative stress is a primary or secondary event in this pesticide-induced neurotoxicity.

5. Pyrethroids

Pyrethroids are a class of synthetic insecticides derived from the naturally occurring pyrethrins isolated from the *Chrysanthemum* genus of plants (Ray and Fry, 2006). Pyrethroids are divided into two classes of compounds based on their toxic signs and structure:

a) Type I or T (tremor) syndrome (i.e. permethrin, allethrin, cimethrin, bifenthrin, bioallethrin) - are devoid of a cyano moiety at the α-position (α-cyano), produce aggressive behavior, fine tremor progressing to whole-body tremor and prostration;

b) Type II or choreoathetosis syndrome (CS) (i.e. deltamethrin, cypermethrin, fenvalerate, cyfluthrin) - possess a α-cyano moiety, produce hypersensitivity, coarse tremor, clonic seizure and profuse salivation (Nasuti et al., 2003) (Table 1).

The main target of pyrethroids-induced neurotoxicity is voltage-gated sodium channels. These insecticides slow the activation or opening of the channels, shifting the voltage dependence of the gates to more hyperpolarized potentials (Clark and Symington, 2012). Therefore, the channel is held open for longer periods, allowing more sodium ions to cross, maintaining a sustained membrane depolarization. Pyrethroids also decrease the opening probability of voltage-gated chloride channels which amplify the sodium channel-mediated signs of intoxication. At relatively high concentrations, deltamethrin and cypermethrin inhibit GABA-gated chloride channels and, as with voltage-gated chloride channels, these effects are specific of type II pyrethroids (Ray and Fry, 2006). These mechanisms are responsible for the observed hyperexcitability of peripheral sites (type I) or central nervous system (type II) in acute poisonings (Table 1).

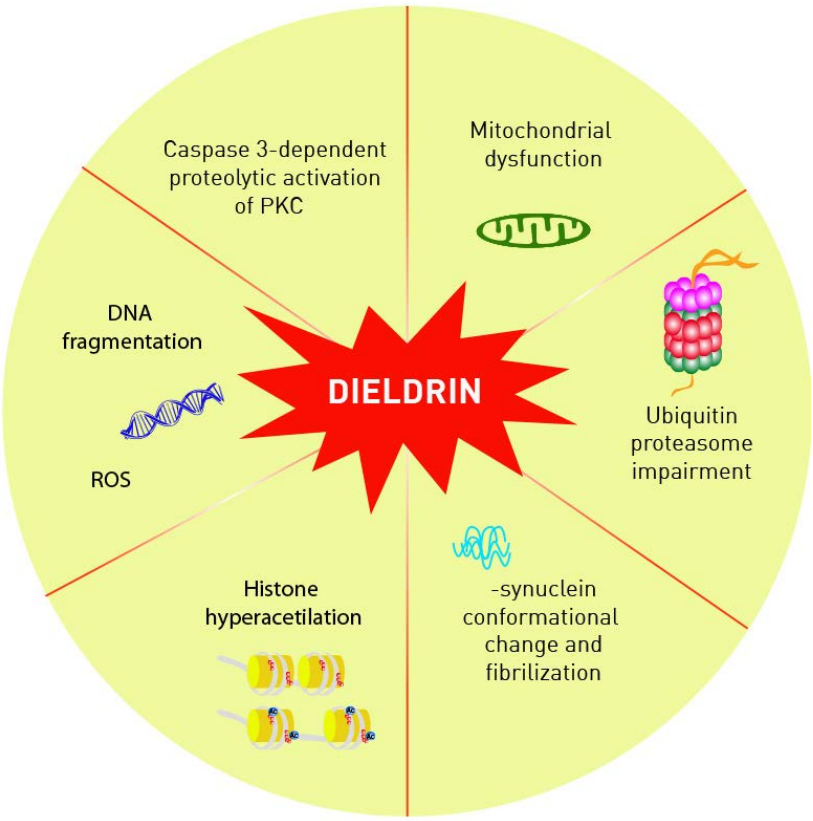

Figure 4. Dieldrin and other cyclodienes are lipophilic compounds and are therefore capable of readily cross the blood brain barrier. Several *in vitro* and *in vivo* studies have shown that dieldrin reproduces many features of Parkinson´s Disease (PD). Dieldrin-induced release cytochrome c is followed by the activation of the caspases cascade and caspase-3-dependent proteolytic activation of protein kinase delta (PKC δ). Dieldrin has the ability to induce mitochondrial dysfunction, oxidative stress, oxidation of DNA, RNA, lipids and proteins. The dysfunction of the ubiquitin proteasome system (UPS) and reduced protein degradation is also responsible for dieldrin´s induction of hyperacetylation of histone, probably due to the accumulation of pivotal histone acetyltransferases. Dieldrin induces α-synuclein aggregation. Together, UPS dysfunction and α-synuclein accumulation enhances the susceptibility of dopaminergic neurons to apoptotic cell death.

Currently, the major concerns of exposure to pyrethroids are developmental neurotoxicity and nigrostriatal dopaminergic neurodegeneration (Shafer et al., 2005; Singh et al., 2012a) (Table 2). Rat pups exposed to deltamethrin 0.7 mg/kg/day over postnatal days 9-13, resulted in a delayed appearance of radial glial fibers, which guide the migration of granule cells of cerebellum (Patro and Patro, 2005). The same group, years later, showed that deltamethrin at the same dose regimen and postnatal period, induced the up-regulation of S100β, a biomarker of brain damage, reduced dendritic arbor with short primary dendrites of purkinje neurons and much reduced stumpy and hypertrophied dendritic branches (Patro et al., 2009). However, the effect of these insecticides in muscarinic receptors, disruption of voltage-dependent sodium channels and other cellular targets, are poorly correlated with the adverse outcomes in adulthood (Ray and Fry, 2006; Shafer et al., 2005).

Low dose of permethrin (0.8-1.5 mg/kg) given to C57 B1/6 mice caused a 33% increase in DA uptake (Karen et al., 2001), similarly with another studies (Bloomquist et al., 2002; Pittman et al., 2003), and a significant increase of DAT protein levels 28 days post treatment (Gillette and Bloomquist, 2003). Unlike DAT, the up-regulation of α-synuclein protein was maximal one day post-treatment and returned to normal levels by the 14- and 28-day (Gillette and Bloomquist, 2003).

Table 1. Summary of the structural and biological differences between class I and II of pyrethroids.

Pyrethroids		
Type I	**Type II**	
Devoided of a cyano moiety at the α-position, produce aggressive behavior, fine tremor progressing to whole-body tremor and prostration (i.e. permethrin, allethrin, cimethrin, bifenthrin, bioallethrin)	Possess a α-cyano moiety, produce hypersensitivity, coarse tremor, clonic seizure and profuse salivation (i.e. deltamethrin, cypermethrin, fenvalerate, cyfluthrin)	
Mechanisms of Neurotoxicity		
Voltage-gated sodium channels	**Voltage-gated chloride channels**	**GABA-gated chloride channels**
Slower activation or opening of the channels, shifting the voltage dependence of the gates to more hyperpolarized potentials. Type I and Type II	Decreased opening probability of the channels which amplify the sodium channel-mediated signs of intoxication. Only Type II pyrethroids	Inhibition of GABA-gated chloride channels. Only Type II pyrethroids

Kou and colleagues showed that 3-month exposure to permethrin (1.5 mg/kg, once a week) had no effect on the expression of TH and DAT protein in striatal dopaminergic terminals, while exposure for longer period (6 months) to either 0.8 mg/kg or 1.5 mg/kg up-regulated TH expression, but did not alter the expression of DAT (Kou and Bloomquist, 2007). Concomitant treatment of permethrin (0.8 mg/kg or 1.5 mg/Kg) with MPTP (20 mg/Kg) for 3 or 6 months did not augment the neurotoxicity of MPTP on the striatal dopaminergic system (Kou and Bloomquist, 2007). Despite some changes were observed across the studies, there is lack of neurodegeneration of dopaminergic neurons after long-term, low-dose exposure to permethrin alone.

Deltamethrin is a type II pyrethroid insecticide, for which the main target is the central nervous system as the compound has little or no peripheral effects common to other pyrethroids. Deltamethrin induces apoptotic cell death in cultured cerebral cortical neurons (Wu et al., 2003), affects different neuronal subtypes in hippocampus, and interferes with cholinergic and dopaminergic neurotransmission mechanisms in different model (Wu and Liu, 2000). Lazarini and colleagues reported that deltamethrin

increases DOPAC levels without changes in DA levels in the adult striatum after prenatal exposure of dams to a non-toxic deltamethrin dose. During their adult life, male rats showed a decreased immobility latency to float and in general activity after the swimming test (Lazarini et al., 2001). Dermal exposure to deltamethrin (30 mg/kg/day, 4 weeks) using an administration schedule mimicking a possible long-lasting occupational skin contamination is accompanied by cerebrocortical injury and loss of hippocampal and striatal DA and DA transporter (Tayebati et al., 2009).

Cypermethrin has been the pyrethroid that has raised more concerns regarding the increase

of risk of developing PD. Singh and colleagues conducted several studies that highlighted the nigrostriatal dopaminergic neurotoxicity of this pesticide (Singh et al., 2011a; Singh et al., 2012a; Singh et al., 2012b; Singh et al., 2011b). Their main findings show that cypermethrin induces neurodegeneration only after long-term exposure (12 weeks) in adult rats and that postnatal pre-exposure enhances the susceptibility, when rechallenged during adulthood (Singh et al., 2012b). Cypermethrin induced-reduction of DA levels, impairment in motor activities and loss of TH^+ cells are microglial activation-dependent (Singh et al., 2011a). Besides microglial activation, other mechanisms of cypermethrin-mediated neurotoxicity have been proposed such as generation of ROS, modulation of antioxidant enzymes and CYP2E1 (Giray et al., 2001; Tiwari et al., 2010) (Table 2).

6. Organophosphates and carbamates

OP are a group of acetylcholinesterase (AChE) inhibitors and represent the largest group of insecticides sold worldwide. Acute OP poisonings leads to the development of three main syndromes: i) acute cholinergic crisis; ii) intermediate syndrome (IMS), and iii) OP-induced delayed polyneuropathy (OPIDP) (Moretto, 1998). Both IMS and OPIDP result of an acute exposure to OP, usually after a suicide attempt or accidental ingestion. The acute cholinergic crisis results from the inhibition of AChE leading to overstimulation of nicotinic and muscarinic receptors in the central and peripheral nervous systems and the consequent signs and symptoms (Lotti, 2001).

IMS is considered a spectrum disorder of the neuromuscular junction that occurs 24–96 h after ingestion of an OP in conscious patients who received treatment for the acute cholinergic syndrome. Respiratory failure associated with IMS is a major contributor to the high morbidity, mortality, and cost of OP poisoning (Abdollahi and Karami-Mohajeri, 2012). The pathophysiology of IMS remains poorly understood although several possible causes such as delayed AChE inhibition, muscle necrosis, down regulation or desensitization of postsynaptic acetylcholine receptors, failure of postsynaptic acetylcholine release, and oxidative stress-related myopathy have been considered to be involved in IMS (Jayawardane et al., 2008; Jayawardane et al., 2009; Yang and Deng, 2007). The toxicokinetics and chemical properties of certain OP critically contribute to the higher probability to develop IMS. For instance, more lipophilic OP are well distributed into fat, leading to a delayed and

prolonged AChE inhibition. Other factors reflect the detoxification of OP such as polymorphisms in *cytochrome P450-paraoxonase 1 (PON1)*, *glutathione S-transferases*, and *cytochromes P450* (Androutsopoulos et al., 2011; Furlong, 2000; La Du et al., 2001; Xiao et al., 2003).

OPIDP is a relatively rare sensory-motor distal axonopathy in humans characterized by degeneration of long axons in the central and peripheral nervous system that appears about 2–3 weeks after exposure or later [reviewed by (Jokanovic et al., 2011)]. The irreversible inhibition of neuropathy target esterase (NTE) is thought to be the main mechanism involved in the pathogenesis of OPIDP (Lotti and Moretto, 2005).

Table 2. Referenced studies in developmental neurotoxicity of pyrethroids.

Model	Compound/ Dose	Major Findings	References
Rat pups PND 0–7 and 9–13	Deltamethrin 0.7 mg/kg/day, i.p.	Up-regulation of S100β. Reduced dendritic arbor with short primary dendrites of purkinje neurons.	(Patro et al., 2009)
Rat pups PND 5-19	Cypermethrin 1.5 mg/kg, 2x week (2 weeks); rechallenged in adulthood 15 mg/kg (2x week,4,8,12 weeks) i.p	Neurodegeneration only after 12 weeks in adult rats. Postnatal preexposure enhances the susceptibility, when rechallenged during adulthood	(Singh et al., 2012b)
Rat pups PND 5-19	Cypermethrin 1.5 mg/kg, 2x week (2 weeks); rechallenged in adulthood 15 mg/kg (2x week,4,8,12 weeks) i.p	Reduction of DA levels, motor dysfunction and loss of TH^+ cells-microglial activation dependent	(Singh et al., 2011a)
Rat pups PND 5-19	Cypermethrin 1.5 mg/kg, 2x week (2 weeks); rechallenged in adulthood 15 mg/kg (2x week,,4,8,12 weeks) i.p	ROS generation, lipid peroxidation and modulation of VMAT 2, CYP2E1, GSTA4-4 expressions	(Tiwari et al., 2010)
Pregnant rats GD 6-15	Deltamethrin 0.08 mg/kg, p.o., once daily	PND 21: no differences in locomotion frequency and immobility duration of male and female; increased male rearing frequency. PND60 males: decreased immobility latency to float; increased DOPAC, DOPAC/DA	(Lazarini et al., 2001)

CYP2E1: cytochrome P450 isoform 2E1; DA: dopamine; DOPAC: 3,4-dihydroxyphenylacetic acid; GSTA4-4: glutathione-S-transferase; PND: postnatal days; ROS: reactiv oxygen species; TH^+: tyrosine hydroxylase positive; VMAT 2: vesicular monoamine transporter 2.

At least 70% of peripheral nerves NTE must be inhibited and subsequently aged to cause the instigation of OPIDP (Johnson, 1990). The irreversible phosphorylation of NTE induces a toxic gain of function by leading to calcium entry, elevation of axonal calpain activity and Wallerian-type degeneration (Glynn, 2000).

Carbamates also act by carbamylating the same site on AChE and NTE, which reversibly inhibits these enzymes activity. Carbamates include insecticides and drugs, such as pyridostigmine and disulfiram. Several clinical cases of polyneuropathy associated with exposure to high levels of carbamates have been reported (Lotti and Moretto, 2006). In three cases, intoxication with methylcarbamates resulted in a clinical and electrophysiological evaluation consistent with peripheral polyneuropathy with distal axonopathy similar to that of OPIDP (Dickoff et al., 1987; Umehara et al., 1991; Yang et al., 2000). More recently, Hu and colleagues reported a case of self- poisoning with a mixture of methomyl–alphamethrin that resulted in cortical blindness and delayed neuropathy (Hu et al., 2010).

Despite the toxic cholinergic effects of OP, there is now substantial evidence that non-cholinergic mechanisms might be associated with the adverse consequences from repeated exposures to low levels of certain OP, such as impairments in attention, memory, and other domains of cognition, as well as chronic illnesses where these symptoms are manifested (e.g., Gulf War Illness, AD) (see reviews, (Androutsopoulos et al., 2013; Terry, 2012)). Chronic low-level exposure to the OP dichlorvos, in adult rats, triggered neuronal apoptosis, elicited an oxidative stress and inflammatory response with impaired mitochondrial complexes I, III and IV activities (Kaur et al., 2007). Binukumar and colleagues also showed that dichlorvos caused nigrostriatal dopaminergic degeneration, reduction in striatal DA and tyrosine hydroxylase levels and positive inclusions for α-synuclein and ubiquitin, resembling the PD features (Binukumar et al., 2010). Others suggested that OP, at doses that were not associated with acute signs of toxicity, can lead to deficits in axonal transport, mitochondrial dynamics (Middlemore-Risher et al., 2011; Terry et al., 2007; Terry et al., 2003) similar to what has been proposed to be involved in the pathogenesis of ALS and AD (Reddy et al., 2012; Shi et al., 2010; Stokin and Goldstein, 2006). Cdk5-dependent hyper-phosphorylation of tau has been considered a biomarker for AD pathology (Maccioni et al., 2001). Chlorpyriphos induced the dysregulation of the D1 receptor/cAMP/PKA signaling pathway, potentiation of corticostriatal glutamatergic transmission, hyperphosphorylation of tau, and induction of aberrant activity of the neuronal protein kinase Cdk5 (Torres-Altoro et al., 2011). As mentioned above, the toxicokinetics of OP significantly contribute to their toxicity. PON1 is an A-esterase which detoxifies several organophosphate-oxons that result from phase-I metabolism of OP such as diazinon, parathion and chlorpyriphos (Costa et al., 2003). There are two main polymorphisms in PON1, one that affects the catalytic site of the enzyme, PON1 192Q/R polymorphism and other, PON1 55L/M polymorphism that is associated with low serum concentration of the enzyme. Importantly, PON1 genotypes might be associated with PD, AD and ALS

(Androutsopoulos et al., 2011; Dardiotis et al., 2013). Carriers of PON1-L55M allele, variant MM genotype and homozygotes for 192R allele possess an increased risk in developing PD (Akhmedova et al., 2001; Manthripragada et al., 2010). However, the data is controversial and several studies have not found an association between PON1 genotypes and PD (Akhmedova et al., 1999; Wingo et al., 2012). The studies focused in the study of the role of PON1 genotypes in AD prevalence have also found positive and negative associations (Androutsopoulos et al., 2011). In a large population of 730 Caucasian and 467 African American AD cases, the authors found a significant association with PON1 S161C/T polymorphism (Erlich et al., 2006). More recently, the groups of Leduc and Erlich have corroborated these results, showing that low levels of PON1 protein, lesser catalytic activity towards paraoxon, and presence of the methionine allele of the 55L/M polymorphism are risk factors for AD (Erlich et al., 2012; Leduc et al., 2009). Morahan and colleagues, found that PON1 promoter polymorphisms were strongly associated with ALS by reducing PON1 expression and possibly modulating the susceptibility of motor neurons to OP (Morahan et al., 2007). As for the other neurodegenerative diseases, despite the epidemiological results demonstrating the association of PON1 and ALS, it is still controversial whether paraoxonases are implicated in this disease pathogenesis (Androutsopoulos et al., 2011).

The main concern regarding the neurotoxicity of OP is related to neurobehavioral changes after long-term exposure to low doses of OP. Recently, Starks and colleagues conducted a study to assess neurobehavioral function in licensed pesticide applicators enrolled in the Agricultural Health Study in Iowa and North Carolina (Starks et al., 2012). The study included 701 male participants and quantitative measures of nine neurobehavioral tests to assess memory, motor speed and coordination, sustained attention, verbal learning and visual scanning and processing. Frequent use of at least one OP pesticide was negatively associated with performance on three of nine neurobehavioral tests and with significantly better performance on six of nine tests. Only malathion was significantly associated with reduced performance on a test of visual scanning and processing (Starks et al., 2012). The inconsistency of association between long-term low or moderate exposure to OP and impaired neurobehavioral function or other neurological effects is seen across several other epidemiological studies (Colosio et al., 2009; Farahat et al., 2003; Kamel and Hoppin, 2004a). The main limitations of the available data are the reduced number of neurobehavioral function tests used per study, and the fact that several studies include cases of a previous acute poisoning to high levels of OP (Roldan-Tapia et al., 2006). Therefore, the observed neurological alterations could be due to an unspecific brain injury, resulting from ischemia/hypoxia, or post-traumatic stress disorder.

Colosio and colleagues reviewed 24 papers published on human neurobehavioral effects of OP and/or carbamates, and found that only 6 papers considered the whole spectrum of functions, the studies yielding positive or uncertain results being 13 for cognitive function, 11 for psychomotor function, 11 for sensory-motor function, and 11 for psychological function impairment. In 46% of

the positive studies a previous severe acute poisoning was reported (Colosio et al., 2009). Another limitation of the studies is the absence of qualitative and quantitative measurement of OP, correlation to AChE activity and neurobehavioral function. Few studies have evaluated these aspects and found positive correlation (Rasoul et al., 2008; Rothlein et al., 2006). In other studies, confounding factors such as exposure to different OP and other pesticides, laboratory methodology, and size of the population, might have led to the poor validity of the cause-effect relationship of OP exposure to the neurobehavioral effects, despite the consistency in the neurobehavioral findings (Rohlman et al., 2011). Even experimental studies in animal models fail to reproduce low dose, long-term exposures to OP. In general, the doses used are sufficiently high to exert signs of acute toxicity and exposure is no longer than 1-3 months. Additionally, neurobehavioral adverse effects only appear when AChE is inhibited (Moser, 2007).

Over the past decade, a growing body of epidemiological and experimental evidence suggests that the oxon metabolites of phosphorothionates insecticides, especially chlorpyriphos (CP) and diazinon, are responsible for the neurodevelopmental toxicity of OP (Bouchard et al., 2011; Flaskos et al., 2007; Flaskos and Sachana, 2010; Rohlman and McCauley, 2010) (Table 3). In different type of primary neuronal or culture cells, CP affected the expression of activated of Ca^{2+}/cAMP response element binding protein (CREB), a transcription factor involved in brain development, and impaired neurite outgrowth, an *in vitro* index of neuronal differentiation (Flaskos et al., 2011; Schuh et al., 2002). CP also decreased the activity of choline acetyltransferase, glutamate decarboxylase, glutamine synthase and cyclic nucleotide phosphohydrolase, biomarkers of neuronal cells, astrocytes and oligodendrocytes, respectively (Monnet-Tschudi et al., 2000). Flaskos and colleagues reviewed several studies revealing that CP and diazinon oxons´s deleterious effects on neuritogenesis are not etiologically related to the inhibition of the enzymatic activity of AChE (Flaskos, 2012). In fact, besides the AChE classical role in synaptic transmission, it also has other 'non-classical' effects such as cell adhesion, shown by the detection of a new class of proteins, the cholinesterase-like adhesion molecules (Paraoanu and Layer, 2008). These adhesion properties are intimately involved in AChE promotion of neurite outgrowth and neural network formation. The suggested mechanism by which AChE might regulate neuritogenesis is associated with its non-catalytic morphogenic activity, protein-protein interactions that may act as a neurite-attractive, as well as network-stabilizing protein during neural development, and neurodegenerative diseases. Alterations in glial cell development results in a higher vulnerability to myelination, synaptic plasticity, and architectural modeling, which is extended until adolescence (Garcia et al., 2002). In glial cells, CP was shown to inhibit cell replication and disrupt cell differentiation. Additionally, CP altered the integrity of the microtubule network and decreased the levels of the microtubule-associated protein MAP 1B and, particularly, tubulin, as well as a reduction in the levels of the cytoskeletal glial fibrillary acidic protein (GFAP) (Garcia et al., 2005). In summary, the above *in vitro* data reveals that

organophosphate-oxons are capable of disrupting separately most phases of nervous system development namely, neuronal cell proliferation, differentiation and apoptosis and glial cells proliferation and differentiation.

Table 3. Referenced *in vitro* studies in long-term exposure to low doses of organophosphates and neurodevelopment.

Model	Concentration/dose	Major findings	References
Fetal rat (DIV 5–15 and DIV 25–35) aggregating cell culture of telencephalon	CP (10^{-8}–10^{-4} M), CP oxon (10^{-10}–10^{-5} M), parathion (10^{-10}–10^{-4} M), paraoxon (10^{-10}–10^{-5} M), during 10 days	Decreased choline acetyltransferase, glutamate decarboxylase, glutamine synthase and cyclic nucleotide phosphohydrolase activities	(Monnet-Tschudi et al., 2000)
Rat pups hippocampal and cortical neurons	CP and CP oxon (0.001-10 μM)	AChE-independent increase of pCREB	(Schuh et al., 2002)
N2a neuroblastoma cells	CP oxon (1–10 μM)	Inhibition of axon outgrowth; reduced levels of protein-43 and NFH	(Flaskos et al., 2011)
PC12 pheochromocytoma and C6 glioma cells	CP and CP oxon (30 μM)	AChE-independent inhibition of DNA synthesis	(Qiao et al., 2001)
N2a neuroblastoma cells	DZ (10 μM)	Inhibition of neurite outgrowth	Flaskos et al., 2007)
C6 glioma cells	CP (5 μg/ml)	Impairment of G-protein signaling, impairment of cell differentiation, reduced expression of the transcription factor Sp1, ↑ROS; the effects were greater in undifferentiated C6 cells but were still detectable in differentiating cells	(Garcia et al., 2001)
C6 glioma cells	DZ oxon (1, 5 and 10 μM)	Decreased GFAP expression, reduced levels of tubulin and MAP1B. Reduced outgrowth of extensions from C6 cells under differentiation-promoting conditions	(Sidiropoulou et al.,2009)
C6 glioma cells	CP and CP oxon (1–10 μM)	Inhibition of the outgrowth of differentiating cells, reduced levels of tubulin and MAP1B	(Sachana et al., 2008)

AChE: acetylcholinesterase; CP: chlorpyriphos; DZ: diazinon; GFAP: Glial fibrillary acidic protein; MAP1B: microtubule associated protein 1B; NFH: neurofilament heavy chain; pCREB: phosphorylated Ca2+/cAMP response element binding protein; ROS: reactive oxygen specie

Bouchard and colleagues, conducted a birth cohort study to assess the association between prenatal and postnatal exposure to OP pesticides, indicated by urinary dialkyl phosphate (DAP) metabolite concentrations, in urine collected during pregnancy and from children at 6 months and 1, 2, 3, 3.5, 5 years of age and cognitive abilities of 7-year-olds (Bouchard et al., 2011). Prenatal, but not postnatal, urinary DAP concentrations were associated with poorer intellectual development in 7-year-old children (Bouchard et al., 2011). Rauh and colleagues reported that prenatal exposure to high levels of CP was associated with higher cognitive deficits evaluated by two different indices, Working Memory Index and Full-Scale IQ in children at 7 years of age (Rauh et al., 2011). Accordingly, Horton and colleagues found that males exposed to CP during the prenatal period were more susceptible to experience a decrement in working memory than females (Horton et al., 2012).

7. Concluding remarks

In humans, pesticides can be responsible for diverse acute and long-term health effects. Even though not consistent, there is a growing body of epidemiologic evidence linking long-term/low-dose pesticide exposure to cancer, reproductive health issues**,** neurodegenerative diseases such as AD, PD, and neurodevelopment impairments in children. Experiments concerning the environmental etiology of PD are more frequent than for other diseases, and several different animal models have been proposed (Cicchetti et al., 2009; Drechsel and Patel, 2008; Moretto and Colosio, 2011). However, a crucial issue is translation for real human exposure to pesticides, tissue concentration reached, and dose regimen used in animal experiments. In these models, a relatively high dose or few consecutive doses of a single compound is usually administered during a short period of time (days or weeks) and the behavioral and/or biochemical analyses are performed within few weeks as well. In contrast, increased health risks are associated with exposure to low levels for several years to decades to a combination of different environmental toxicants. Therefore, there is an urgent need to standardize the doses, age, species or strain, duration of treatment and the methodology to assess neurodegeneration.

Particularly, PQ and MB exposure has been largely associated with PD. Other pesticides such as rotenone, dieldrin and diquat have also been shown to reproduce some features of PD in animal models. However, no single compound, including the non-pesticide MPTP, is able to reproduce all the hallmarks of human PD (Blesa et al., 2012; Cicchetti et al., 2009). Combined exposure to PQ+MB, or MPTP+PQ/MB yields potentiated damage to dopaminergic system, producing cell damage and loss, even when the doses of each compound are non-toxic. Most likely, PD might result from a prolonged contact to sub-toxic multi-hits at different targets within the dopaminergic system.

Despite the numerous studies, association between neurobehavioral adverse effects and OP is only consistent regarding subjects acutely poisoned, instead of the uncertainty in data concerning subjects chronically exposed to low doses of OP. Because of the complexity of the effects of environmental exposures on human health, the current

available data do not support a good correlation between actual pesticide exposure and development of PD or other neurodegenerative diseases. Further research should focus on the improvement of the characterization of exposure in epidemiological studies (pesticide identification and quantification), particularly the categorization of previously acute poisoned subjects and prevalent/incident cases. Future investigations should also concentrate in designing animal studies that better simulates human exposure and measures the same aspect of neurological function and outcomes.

Conflict of interest

The authors declare that there are no conflicts of interest.

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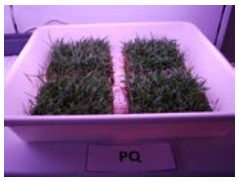
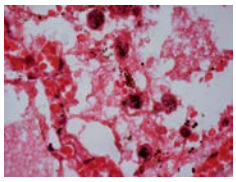
CHAPTER III

GENERAL AND SPECIFIC OBJECTIVES

Taking into consideration the above-mentioned regarding the burden of PQ poisoning, the increasing pressure from government and regulatory agencies to reduce the pesticide poisoning, and the current inexistence of a safe formulation, the global aim of this thesis was to develop a safer PQ formulation with the incorporation of LAS as the antidote, preserving the herbicide effectiveness and maintaining a low environmental impact.

The strategy pursued to achieve the proposed main objective comprised the following original studies:

Study I

(i) To test if the incorporation of LAS in the standard PQ formulation (Gramoxone® 20%) improves the survival rate in intoxicated male Wistar rats.

(ii) To assess the herbicidal activity of the new formulation, PQ+LAS.

(iii) To determine the stability of the new formulation regarding the herbicidal effect and toxicity to rats.

Study II

(i) To evaluate the toxicity of the PQ+LAS formulation to primary producers. The toxicity of the new formulation was compared to the standard product concerning the toxicity against the species of microalga *Chlorella vulgaris*.

Study III

(i) To evaluate the acute oral toxicity of this new formulation following an up-and-down protocol (UDP).

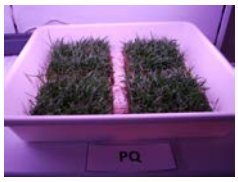
(ii) To evaluate the acute toxicity of the new formulation PQ+LAS***.***

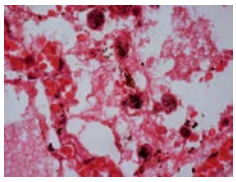
(iii) To study LAS influence in PQ toxicokinetics.

(iv) To clarify the mechanisms involved in the protective effect of LAS

CHAPTER IV

ORIGINAL RESEARCH

Study I

New formulation of paraquat with lysine acetylsalicylate with low mammalian toxicity and effective herbicidal activity

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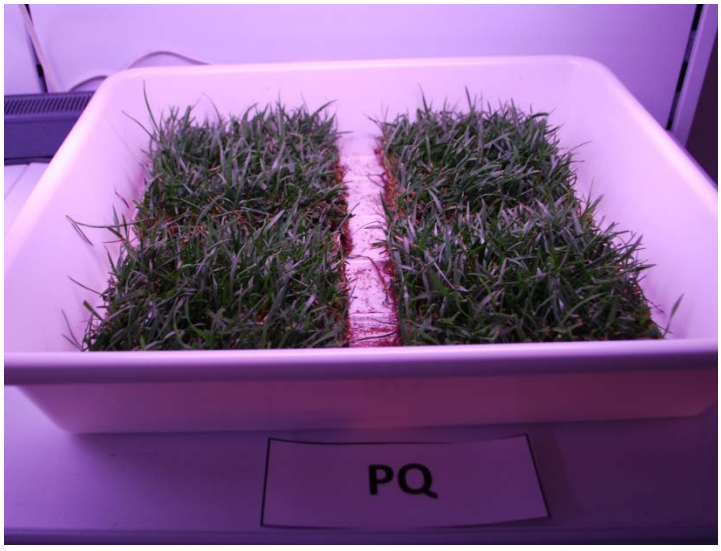

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New formulation of paraquat with lysine acetylsalicylate with low mammalian toxicity and effective herbicidal activity†

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Abstract

BACKGROUND: Currently, the commercial formulations of the herbicide paraquat are highly toxic to humans, and no effective antidote is available for paraquat poisoning. The aim of the present study was to develop a safe formulation, combining paraquat and the known antidote lysine acetylsalicylate. The toxicity of a mixture of Gramoxone® (20% paraquat) and lysine acetylsalicylate in adult Wistar male rats and the herbicidal efficacy against grass lawn (50% of *Poa pratensis* and 50% of *Festuca arundinacea*) were evaluated. This new formulation was administered to Wistar rats by gavage at 125 mg kg^{-1} of paraquat ion and lysine acetylsalicylate at 79, 158 or 316 mg kg^{-1} body weight, and the survival rate was observed for 30 days.

RESULTS: The survival rate of the paraquat group was only 40%, while lysine acetylsalicylate provided effective protection, with full survival observed in the groups that received 125 mg kg^{-1} of paraquat ion and 316 mg kg^{-1} of lysine acetylsalicylate. Both formulations of paraquat, either in the absence or in the presence of lysine acetylsalicylate, provided the same herbicidal activity against the tested herbal species.

CONCLUSIONS: The present formulation of paraquat containing lysine acetylsalicylate, significantly decreases mammalian toxicity while maintaining effective herbicidal activity.

Keywords: paraquat; antidote; lysine acetylsalicylate; herbicide

1 INTRODUCTION

The continued growth of the human population requires an increased food supply that needs to be obtained in a sustainable way. This implies the adoption of manageable agriculture practices, maintaining or improving crop yields on existing farmed land while avoiding expansion of agricultural activities into remaining areas of natural vegetation. One of the crucial factors is often weed control, which requires effective and environmentally friendly herbicides, as mechanical control is either difficult with machinery or very laborious when done by hand, frequently resulting in soil erosion and lower productivity.[1]

Paraquat (PQ) (1,1′-dimethyl-4,4′-bipyridilium dichloride) is one of the leading herbicides used in no-till farming methods that were developed to reduce soil erosion and reduce labour and fuel costs.[2] The adoption of direct drilling planting methods using PQ has also demonstrated other environmental benefits, including preservation of soil organic matter, soil aeration, edaphic fauna, structure and functioning, prevention of soil erosion and reduction in production of methane gas.[1]

PQ is non-systemic, as it is only slightly translocated within plants, partially because of the rapid desiccation of the green tissues, and so underground parts such as tubers and roots are not affected and usually regrow.[3] This would be a disadvantage of the long-term control of perennial-weed herbicide, but for PQ it represents a real advantage in terms of crop plant protection when sprayed accidentally because only the green part receiving the herbicide is affected.[4] Herbicidal activity becomes evident through rapid appearance of a brownish colour and the desiccation of

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green plant tissue following exposure to direct sunlight. Indeed, bipyridylium quaternary ammonium herbicides, such as PQ, are activated by light and oxygen and express their phytotoxicity quite rapidly, depending of the photosynthetic rate of the plant.[5] Interception of electron flow from photosystem I, by PQ, essentially shunts the electron transport chain, leading to an inhibition of oxidised nicotinamide adenine dinucleotide phosphate reduction during photosynthesis.[6] By accepting one electron, PQ is reduced to the paraquat monocation free radical ($PQ^{\bullet+}$), which is rapidly reoxidised by O_2 to PQ^{2+}, initiating a series of reactions leading to production of superoxide anion ($O_2^{\bullet-}$) with subsequent cell membrane disruption and plant death.[7]

Unfortunately, suicide attempts with PQ and other pesticides are a major public health problem in developing and developed countries, causing a huge number of deaths.[8,9] In South Korea, from 1996 through 2005, PQ was the most frequent cause of pesticide fatal poisoning, accounting for 538 (35.5%) of all pesticide-related deaths,[10] and, in Trinidad and Tobago between 1986 and 1990, 63% of all suicide deaths were due to PQ.[11]

In self-poisoning, PQ is usually taken orally and absorbed through the gastrointestinal tract, inducing toxicity through redox cycling and consequent generation of deleterious ROS.[12] Although organophosphates account for the majority of pesticide poisonings, the very high fatality rate (>50%) of PQ poisonings makes it the foremost single pesticide causing death in many countries, including Sri Lanka,[13] South Trinidad,[11] Samoa[14] and South Korea.[10,15] Even though the major cause of intoxication is intentional self-poisoning,[16] accidental ingestion of diluted paraquat, as well as occupational poisoning, through skin absorption from a leaking backpack, have been reported.[17–19]

In spite of attempts to develop more effective therapeutics, no successful effective treatment has been found so far.[20] An alternative approach to lowering PQ toxicity to mammals is to modify the PQ formulation. In attempt to reduce toxicity, the formulation of PQ has been modified several times since its introduction in the market. Firstly, a blue dye, a stenching agent and an emetic were added to the liquid concentrate in the late 1970s.[21] In 2004, a new PQ formulation designed to reduce its toxicity was introduced by Syngenta in Sri Lanka, under the trade name Inteon®.[22] This new formulation included three components designed to reduce PQ absorption: (i) an alginate to thicken the formulation in the acidic environment of the stomach; (ii) an increase in the amount of emetic to induce vomiting more quickly; (ii) a purgative to speed its elimination from the small intestine, which is the main site of its absorption.[22] Although this survey has shown that Inteon® reduces the mortality of patients following PQ ingestion and increases survival time, a more recent study has shown that the beneficial effect associated with the Inteon® formulation could not be sustained.[23] Therefore, in spite of the slight enhancement of survival, the existing formulations of PQ in the market are still highly toxic.

In the past few years, the authors' research group has focused on the development of new antidotes to PQ poisoning. In previous studies it was demonstrated that sodium salicylate (NaSAL) has a great potential to be used as an antidote against PQ-induced lung toxicity, through an effective inhibition of pro-inflammatory factors such as nuclear factor kappa-B (NF-κB), scavenging of ROS, inhibition of myeloperoxidase activity, inhibition of platelet aggregation and by preventing death of pulmonary cells through apoptotic pathways.[24,25] Importantly, this treatment was associated with full survival of PQ-intoxicated rats. Subsequently, lysine acetylsalicylate (LAS), which releases salicylate *in vivo* and is available in hospitals for parenteral administration, was administered 2 h after PQ poisoning and proved to assure full survival of the animals[26] It is noteworthy that the incorporation of LAS in PQ formulation will overcome the disadvantage of the critical time between intoxication and treatment, with an expected increase in survival rate and decrease in morbidity. Although the mixing of PQ with an antidote appears to be a logical approach, there are no data showing that LAS or PQ would maintain its properties in a mixture, i.e. be effective as an antidote or herbicide respectively. Therefore, the overall aim of this study was to develop a safer PQ formulation with the incorporation of LAS as the antidote and preserving the herbicide effectiveness. A further objective was to determine the stability of the formulation.

2 MATERIAL AND METHODS

2.1 Chemicals and drugs

The commercial formulation of PQ used was Gramoxone® [20% PQ (1,1′-dimethyl-4,4′-bipyridilium dichloride) hydrate; molecular mass 257.2 g mol^{-1}], kindly supplied by Syngenta Crop Protection Lda (Lisboa, Portugal). Ampoules of LAS (molecular mass 326.35 g mol^{-1}) and water for injections were a generous gift from Labesfal Genéricos (Campo de Besteiros, Portugal). The formulation administered corresponds to the same available for human treatment in Portugal. The plant material was purchased from Flor do Norte (Arcozelo, Portugal).

2.2 Animals

Adult male Wistar rats (aged 8 weeks) obtained from Charles River SA (Barcelona, Spain), with a mean body weight of 221 ± 21 g, were used. Animals were kept in standard laboratory conditions (12/12 h light/darkness, 22 ± 2 °C room temperature, 50–60% humidity) for at least 1 week before starting the experiments. Animals were allowed to have free access to tap water for human consumption and rat chow *ad libitum* during the quarantine period. Animal experiments were approved by the Portuguese Agency for Animal Welfare (General Board of Veterinary Medicine in compliance with the Institutional Guidelines and the European Convention).

2.2.1 *Evaluation of survival rate*

For the evaluation of survival rate, after the quarantine period, 25 animals were randomly divided into five groups of five animals each. They were maintained in groups of five animals per cage. Tap water and rat chow were given *ad libitum* during the entire experiment. The PQ administered dose (125 mg kg^{-1} of PQ ion) corresponds to the previously reported oral LD_{50}.[26–28] Based on previous work,[26] the approach was to mix the commercial formulation of PQ (Gramoxone®) with increasing concentrations of LAS. The molar proportions between PQ and LAS were 1:0.5; 1:1 and 1:2. The PQ + LAS mixture was diluted in distilled water, as both compounds are water soluble. The administration of the vehicle (distilled water), the PQ and the PQ + LAS mixture was done by gavage in a volume of 0.5 mL per 250 g body weight. The five groups were treated as follows (given doses were per kg body weight): (i) control group, $n = 5$, animals treated with distilled water; (ii) PQ group, $n = 5$, animals exposed to Gramoxone® PQ (125 mg kg^{-1}); (iii) PQ + LAS (1:0.5), $n = 5$, animals exposed

to the mixture of PQ (125 mg kg^{-1}) + LAS (79 mg kg^{-1}); (iv) PQ + LAS (1:1), $n = 5$, animals exposed to the mixture of PQ (125 mg kg^{-1}) + LAS (158 mg kg^{-1}); (v) PQ + LAS (1:2), $n = 5$, animals exposed to the mixture of PQ (125 mg kg^{-1}) + LAS (316 mg kg^{-1}). For assessment of formulation stability as regards safety, ten additional animals were divided into two groups, and the survival rate was monitored after treatment with the same formulation that resulted in full survival [PQ (125 mg kg^{-1}) + LAS (316 mg kg^{-1})], but in this case the formulation had 2 and 6 months of shelf life. Body weight, motor activity, dyspnea, nasal haemorrhage and hair characteristics were noted and recorded if present, and survival was monitored for 30 days.

2.3 Assessment of herbicidal efficacy

2.3.1 Controlled environment chamber experiment

Herbicidal efficacy was assessed in a grass lawn consisting of a mixture of 50% *Poa pratensis* (smooth meadow grass, common meadow grass or Kentucky bluegrass) and 50% *Festuca arundinacea* (*Lolium arundinaceum*; *Schedonorus phoenix*). The lawns were divided into 14 cm × 14 cm squares and were randomly separated into four groups of four squares each. Every group of four squares was placed in a rectangular tray with water in the bottom. The grass was maintained in a ventilated room at 18.7 ± 0.2 °C with a humidity of 69.8 ± 5.9% and illuminated with cool-white fluorescent lights at a photoperiod light intensity of 61.3 ± 5.3 µE m^{-2} s^{-1} (16:8 h light/dark cycle). In all chemical applications, the grass was sprayed with a hand sprayer with a volume sufficient to ensure a complete cover (medium volume 6 mL). The sprayed area was limited with a plastic funnel of 8 cm diameter, and the distance of application was maintained as well as the pressure applied. All exposures containing both PQ and LAS or PQ or LAS alone were diluted and applied as soon as possible after mixing. The commercial formulation was diluted according to the manufacturer's instructions (Syngenta Crops, Lda), mixing 100 parts of water for each part of product (final concentration 1%).

The four grass groups were treated as follows: (i) control group, $n = 4$, grass recieved only water; (ii) LAS group, $n = 4$, grass exposed to LAS (5.1 g L^{-1}); (iii) PQ group, $n = 4$, grass exposed to PQ (2 g L^{-1}); (iv) PQ + LAS 0 months group, $n = 4$, grass exposed to PQ (2 g L^{-1}) + LAS (5.1 g L^{-1}); (v) PQ + LAS 2 months group, $n = 4$, grass exposed to PQ (2 g L^{-1}) + LAS (5.1 g L^{-1}) with 2 months of shelf life; (vi) PQ + LAS 6 months group, $n = 4$, grass exposed to PQ (2 g L^{-1}) + LAS (5.1 g L^{-1}) with 6 months of shelf life. The experiment was monitored for 6 days, and the herbicidal activity was determined by visual assessment and expressed as percentage of necrotic (yellow-brownish colour) and desiccated (loss of water) area. For that purpose, the desiccation and necrosis effects were semi-quantified by analysing four images of each group using digital photo software (Irfanview, ImageJ, Image Analyzer).

2.4 Statistical analysis

All statistical calculations were performed with GraphPad Prism v.5.00 for Windows (GraphPad Software, San Diego, CA). Comparison of the survival curves was performed using the logrank test. Data obtained from the results of the herbicidal activity experiments were expressed as mean ± SD (standard deviation). Statistical comparison of different treatments was done by two-way ANOVA on ranks followed by Bonferroni's *post hoc* test. In all cases, *P* values lower than 0.05 were considered as statistically significant.

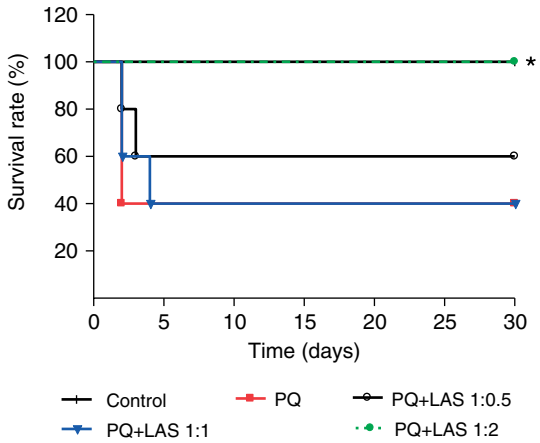

Figure 1. Survival rate of Wistar rats following gavage administration of water (control), paraquat (PQ) and PQ plus lysine acetylsalicylate (LAS) groups: (PQ + LAS 1:0.5; PQ + LAS 1:1; PQ + LAS 1:2) groups. * $P < 0.05$ for PQ versus PQ + LAS 1:2 group.

3 RESULTS

3.1 Survival rate

Rats exposed to Gramoxone® (PQ group) only displayed 40% survival at the end of 30 days (Fig. 1). All deaths occurred within 48 h in the PQ-intoxicated group. The concomitant administration of PQ and LAS in different proportions elicited different results. In the PQ + LAS 1:0.5 group, 60% survival was observed, while in the group that received a higher dose of LAS (PQ + LAS 1:1) only 40% survival was registered, but this variation is not statistically significant, which indicates that both doses are ineffective. However, for the highest dose of LAS (PQ + LAS 1:2), an increase in the survival rate to 100% was obtained. During the monitored period of time, the PQ + LAS 1:2 group showed only mild signs of apnea within 48 h of poisoning and recovered completely after 72 h. All the rats intoxicated with similar solutions but with 2 and 6 months of shelf life survived (100% survival), showing moderate signs of injury, smooth nose haemorrhage and reduced activity only in the first 48 h, and their condition ameliorated during the follow-up time (30 days).

3.2 Assessment of herbicidal efficacy

3.2.1 Controlled environment chamber experiment

The area of grass sprayed with PQ (2 g L^{-1}) became slightly desiccated within 24 h of application and became more necrotic in the following days, reaching the maximum effect after 6 days (Fig. 2). The addition of LAS (5.1 g L^{-1}) to the foliar spray solution (concomitant application) showed no differences from the PQ group at the end of the experiment (Fig. 2). Simultaneous foliar application of PQ and LAS provided similar efficacy to PQ alone. Indeed, in both groups the damage was time dependent, and the first signs of desiccation were observed within 24 h (Fig. 3). At the end of the experiment (6 days after application) the results were similar in both groups. In the experiments carried out with two- and six-month-old formulations, the herbicidal activity was maintained. However, the efficacy showed a significant decrease ($P < 0.01$ for PQ versus PQ + LAS 2 months, and $P < 0.001$ for PQ versus PQ + LAS 6 months) in the desiccation and necrosis percentage by comparison with the PQ group (Fig. 3). LAS treatment (5.1 g L^{-1}) did not show any phytotoxicity under the conditions of this study.

Figure 2. Foliar application of control (water), lysine acetylsalicylate (LAS) (5.1 g L^{-1}), paraquat (PQ) (2 g L^{-1}) and PQ + LAS (2 g L^{-1} + 5.1 g L^{-1}) solutions in grass lawn (50% of *Poa pratensis* and 50% of *Festuca arundinacea*). Simultaneous application of PQ + LAS showed similar herbicidal activity to PQ alone. No phytotoxicity was observed in the control and LAS group. The sprayed area is limited by the dashed white line. Plants were photographed every 2 days before the next foliar application.

4 DISCUSSION AND CONCLUSION

The results obtained in the present study clearly demonstrate that LAS, in a dose of 316 mg kg^{-1}, concomitantly administered by gavage with 125 mg kg^{-1} of PQ, prevented PQ-induced toxicity and mortality in Wistar rats without affecting the herbicidal efficacy of PQ. Thus, for the first time it was shown that the inclusion of an antidote in the PQ commercial formulation (Gramoxone®) was capable of improving the survival rate of treated Wistar rats. These findings corroborate previous results showing that both NaSAL and LAS were effective antidotes when administrated 2 h after PQ poisoning.[25]

In the past few decades, a considerable and consistent amount of evidence has demonstrated that LAS and its metabolite salicylic acid (SA) have antioxidant properties. ASA possesses antioxidant activity by scavenging the hydroxyl radical and chelation of transition metals, stimulation of NO synthesis, increased expression of lipoxins, inhibition of neutrophil oxidative burst, inhibition of NF-κB, activator protein 1 and protein kinases[24,25] and inhibition of the lectin-like oxidised LDL receptor-1.[29] These effects are possible mechanisms underlying the observed protective effect of LAS against PQ-induced toxicity and may contribute to the success of this novel formulation. Although it is not the most common pesticide used in suicide attempts, PQ has a higher incidence of fatalities than other common pesticides.[30] The problem of intentional or accidental ingestion of PQ encouraged the introduction of formulation changes (a blue colour, a stenching agent and an emetic) to the concentrated formulation.[21] However, the value of these measures is still questionable since the mortality rate after intoxication remains high,[13,31] unintentional PQ ingestion being the only etiology of exposure that has decreased in the past few years.[17,21] Several detoxification strategies aiming to prevent gastrointestinal absorption, such as gastric lavage and the administration of mineral absorbents (activated charcoal, Fuller's earth and bentonite), have been used with limited efficacy; for example, gastric lavage remains controversial and should not be used without administration of an adsorbent.[32–35] The target of recent research on new PQ formulations has been the reduction of PQ gastrointestinal absorption through the incorporation of an alginate that forms a gel in the acidic environment of the stomach, limiting the absorption through the epithelia of the small intestine.[36] Nevertheless, efforts to reduce the absorption of PQ did not improve the survival rate following accidental or intentional ingestion of PQ-containing agricultural products.[23] The incorporation of a molecule with a broad spectrum of activity and pleiotropic functions, such as LAS, might reduce the high mortality rate associated with PQ poisoning.

In order to obtain a product entirely compatible with commercial needs, the herbicidal effect of the novel formulation was also

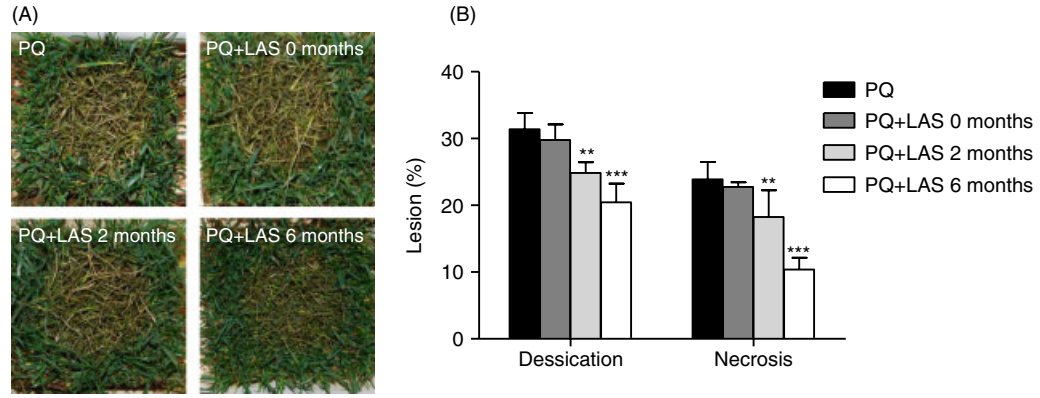

Figure 3. (A) PQ: grass sprayed with the commercial available formulation of PQ (2 g L^{-1}); PQ + LAS 0 months: grass sprayed with a freshly prepared PQ + LAS (2 g L^{-1} + 5.1 g L^{-1}) formulation; PQ + LAS 2 months: grass sprayed with a PQ + LAS (2 g L^{-1} + 5.1 g L^{-1}) formulation with 2 months of shelf life; PQ + LAS 6 months: grass sprayed with a PQ + LAS (2 g L^{-1} + 5.1 g L^{-1}) formulation with 6 months of shelf life. (B) Percentage of necrosis and desiccation after foliar application of paraquat (PQ) (2 g L^{-1}), PQ + lysine acetylsalicylate (LAS) (2 g L^{-1} + 5.1 g L^{-1}) fresh solution (PQ + LAS 0 months) and PQ + LAS with 2 and 6 months of shelf life. ** $P < 0.01$ for PQ versus PQ + LAS 2 months; *** $P < 0.001$ for PQ versus PQ + LAS 6 months.

assessed in the present study against grass species belonging to the *Poaceae* family. The species of this family are included in OECD guidelines as possible test species for use in plant toxicity testing.[37] In the present experimental model, the PQ + LAS solution showed the same efficacy as the standard PQ formulation. Grass leaves became slightly desiccated within 24 h of application of both solutions. Some authors claim a 3 h PQ-induced toxicity with similar concentrations applied, although the light intensities employed were substantially different, 250 μE m^{-2} s^{-1} as opposed to 61.3 ± 5.3 μE m^{-2} s^{-1} used in the present study.[38] PQ is activated by sunlight, so it is expected that, with increasing light intensity, the damage to the leaves will increase as well. The present results showed that, even with a lower light intensity, PQ induced a significant damage at day 6 (Fig. 2). A key consideration here is the compatibility of the PQ (200 g L^{-1}) + LAS (510 g L^{-1}) mixture, as LAS has good water solubility.

Importantly, LAS does not chemically react with PQ or antagonise PQ herbicidal activity. Actually, SA, the main metabolite of LAS, forms a soluble crystalline charge-transfer (CT) complex with PQ dichloride.[39] This was first hypothesised on the basis of the chemical behaviour of SA, which is similar to that of polyphenols containing a free hydroxyl group.[40] In view of the fact that LAS is acetylated in that position, it is unable to form a CT complex with PQ, which represents the most probable reason by which herbicidal effectiveness is maintained. Another advantage of LAS is that, *in vivo*, 50% is immediately hydrolysed (de-acetylated) to SA in the gastrointestinal mucosa, and the remaining 50% is hydrolysed within 15 min of gastric and intestinal absorption. On the one hand this pharmacokinetic behaviour may allow complex formation between PQ and SA in the stomach, thus preventing its absorption, and on the other hand the remaining PQ absorbed may be systemically complexed with SA. This putative mechanism of complexation with PQ *in vivo* might also contribute to the safer profile of LAS.

The new formulation developed showed an improved safety profile, as demonstrated by the full survival rate of male rats intoxicated with this formulation. Importantly, in addition to the experiments performed immediately after the preparation of the formulation, 100% survival was also recorded in animals treated with two- and six-month-old formulations (that is, after their preparation), indicating a good protective efficacy of the formulation in the long term. In spite of the excellent results concerning the mammalian toxicity of the six-month-old formulation, the herbicidal activity was weaker at the end of the experiment by comparison with the efficacy of the freshly prepared solution. This partial loss of activity could be explained either by increased hydrolysis of LAS into SA with time, as it is known that SA can interfere with the herbicidal effect of PQ, or by the degradation of PQ itself.[38]

All the formulations containing PQ were banned from use in the European Union in 2007. Among other reasons, concerns about potential links between PQ and Parkinson's disease and the requirement of human protection, which prohibits any exposure higher than the acceptable operator exposure level, as well as the protection of animal health, dictated the ban.[41] Most of all, its high toxicity and lack of antidote contributed to this decision. Nevertheless, PQ is still used in the United States, in South America and in Asia in countries like Malaysia, India, Vietnam and China. After nearly 50 years on the market, farmer demand for PQ continues to grow, with weed resistance to glyphosate and soil conservation needs being important aspects. The present study shows that this new formulation has the potential to reduce the risks associated with PQ intoxication in mammals, and thus might considerably improve the safety of the product. Nevertheless, these results are preliminary, and further work is necessary to assess the formulation stability, the full scale-up costs and the development of a technology able to prevent LAS hydrolysis within the formulation.

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Study II

Lysine acetylsalicylate increases the safety of a paraquat formulation to freshwater primary producers: A case study with the microalga *Chlorella vulgaris*

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Lysine acetylsalicylate increases the safety of a paraquat formulation to freshwater primary producers: A case study with the microalga *Chlorella vulgaris*

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ABSTRACT

Large amounts of herbicides are presently used in the industrialized nations worldwide, with an inexorable burden to the environment, especially to aquatic ecosystems. Primary producers such as microalgae are of especial concern because they are vital for the input of energy into the ecosystem and for the maintenance of oxygen in water on which most of other marine life forms depend on. The herbicide paraquat (PQ) is known to cause inhibition of photosynthesis and irreversible damage to photosynthetic organisms through generation of reactive oxygen species in a light-dependent manner. Previous studies have led to the development of a new formulation of PQ containing lysine acetylsalicylate (LAS) as an antidote, which was shown to prevent the mammalian toxicity of PQ, while maintaining the herbicidal effect. However, the safety of this formulation to primary producers in relation to commercially available PQ formulations has hitherto not been established. Therefore, the aim of this study was to evaluate the toxicity of the PQ + LAS formulation in comparison with the PQ, using *Chlorella vulgaris* as a test organism. Effect criterion was the inhibition of microalgal population growth. Following a 96 h exposure to increasing concentrations of PQ, *C. vulgaris* growth was almost completely inhibited, an effect that was significantly prevented by LAS at the proportion used in the formulation (PQ + LAS) 1:2 (mol/mol), while the highest protection was achieved at the proportion of 1:8. In conclusion, the present work demonstrated that the new formulation with PQ + LAS has a reduced toxicity to *C. vulgaris* when compared to Gramoxone®.

1. Introduction

A considerable amount of the herbicides applied in crop fields enters into freshwater aquatic ecosystems through surface runoff or leaching, among other processes, leading to environmental contamination and toxic effects potentially harming the aquatic organisms (Tornqvist et al., 2011). This phenomenon has motivated great concerns, especially regarding the toxicity towards the phytoplankton, and microalgae in particular. Microalgae, as major primary producers, are essential for the equilibrium of the aquatic ecosystems, and therefore adverse effects of pollutants on these organisms may have reflexes in the whole ecosystem, potentially compromising its biodiversity, structure and function (Campanella et al., 2001), and the services that it provides.

Among herbicides, PQ (1,1′-dimethyl-4,4′-bipyridilium dichloride) is responsible for the highest human morbidity and mortality worldwide, being responsible for several hundred thousand deaths

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since its introduction; for this reason its use has been fiercely discouraged, and even banned in several countries (Baltazar et al., 2013b). Despite its mammalian toxicity, PQ has an environmental risk lower than other herbicides, in great part due to its reduced leaching or surface runoff, attributable to its rapid deactivation in the soil (Bromilow, 2004). Nevertheless, microalgae are very sensitive to PQ and, consequently, even small amounts of herbicide reaching the aquatic environment can still be toxic (Ibrahim, 1990; Ma et al., 2002, 2004, 2006; Saenz et al., 1997). PQ inhibits the photosynthesis in green algae by accepting electrons from photosystem I leading to an inhibition of oxidized nicotinamide adenine dinucleotide phosphate reduction (Qian et al., 2009). PQ is then reduced to the paraquat monocation free radical ($PQ^{\bullet+}$), which is rapidly reoxidized by O_2 to PQ^{2+}, initiating a series of reactions leading to the production of superoxide anion ($O_2^{\bullet-}$) with subsequent cell membrane disruption and cell death (Dinis-Oliveira et al., 2008b; Zweig et al., 1965).

Microalgae are extremely useful as biosensors and as tools to assess the toxicity of hazardous substances in water (Ma et al., 2004). In addition, their short generation times and rapid responses to environmental changes have made these toxicity tests highly valuable for comparison purposes (Ma et al., 2002; OEDC, 2006). For these reasons, studies designed to evaluate aquatic toxicity frequently use microalgae, rather than macrophytes as test organism. In particular, *Chlorella vulgaris* is the species of microalgae most commonly used to represent primary producers.

The toxicity tests in algae typically evaluate population-based parameters, such as the specific growth rate, biomass, cell yield, and chlorophyll fluorescence (Prado et al., 2009). Previous studies reported variable EC_{50} values for PQ, ranging from 0.2 μg/L to 140 μg/L, in a 96 h growth inhibition test carried out with the green microalga *C. vulgaris* (Ma et al., 2002; Saenz et al., 1997). The acute toxicity of PQ, on three other microalgae (*Scenedesmus dimorphus*, *Scenedesmus quadricauda* and *Ankistrodesmus falcatus*) was also previously studied, showing that *Scenedesmus dimorphus* was the most susceptible among the species studied (Ibrahim, 1990). More recently, other authors reported the effects of PQ on parameters other than growth or biomass, namely cellular viability, metabolic activity as given by flow cytometry, DNA damage, measurement of reactive oxygen species (ROS), and expression profile of genes involved in oxidative stress defense mechanisms, such as L-ascorbate peroxidase, glutaredoxin and glutathione-*S*-transferase related genes (Jamers and De Coen, 2010; Prado et al., 2009; Qian et al., 2009).

Previous studies have shown that the antioxidant and anti-inflammatory properties of salicylates, namely, salicylic acid (SA), the metabolite of acetylsalicylic acid and lysine acetylsalicylate (LAS), proved to be useful as antidotes against PQ toxicity (Dinis-Oliveira et al., 2007a, 2007b, 2009a, 2009b). These studies culminated in the development of a new formulation of PQ containing lysine acetylsalicylate as an antidote, which was shown to prevent the mammalian toxicity of PQ, while maintaining the herbicidal effect (Baltazar et al., 2013a). However, the safety of this new formulation to primary producers has not yet been established. Therefore, the aim of this study was to evaluate the toxicity of the PQ/LAS formulation in comparison with the PQ, using *C. vulgaris* as the test organism.

2. Material and methods

2.1. Chemicals and drugs

The commercial formulation of paraquat used was Gramoxone® [20% (w/v) PQ (1,1′-dimethyl-4,4′-bipyridinium dichloride hydrate; molecular mass = 257.2 g/mol], kindly supplied by Syngenta Crop Protection Lda (Lisboa, Portugal). Ampoules of LAS (molecular mass = 326.35 g/mol) were a generous gift from Labesfal Genéricos (Campo de Besteiros, Portugal).

2.2. Chlorella vulgaris culture conditions

The green alga *C. vulgaris* was used as test organism. Cultures have been maintained at 20 ± 1 °C with continuous aeration and continuous (24 h) photoperiod provided by solar spectrum cool fluorescent light with an intensity of 75–80 μE/m^2/s for several generations in the Laboratory of Ecotoxicology and Ecology of CIIMAR and Laboratory of Ecotoxicology of ICBAS, University of Porto. *C. vulgaris* cultures were maintained in exponential growth in 5L Erlenmeyer flasks containing 4 L of liquid MBL medium (Stein, 1973) partially renewed every two days. The culture medium and all the glass material was sterilized at 121 °C for 1 h prior to use.

2.3. Growth inhibition test

2.3.1. Paraquat formulation toxicity assays

C. vulgaris toxicity test was conducted for 96 h following the Organization for Economic Co-operation and Development (OECD) Guideline 201 "Freshwater Alga and Cyanobacteria, Growth Inhibition Test" (OEDC, 2006). All treatment and control experiments were prepared from the *C. vulgaris* culture and each 400 mL Erlenmeyer flask was inoculated with exponentially growing cultures, to achieve an initial cell density of 1×10^4 cells mL. For the evaluation of PQ toxicity, each flask of media (400 mL) containing *C. vulgaris* was then exposed to Gramoxone® (PQ solution) diluted to concentrations of 20, 40, 80, 160, 320, 640 and 1280 μg/L, except the control that was not exposed to the PQ formulation (Saenz et al., 1997). Three replicates from three independent assays (3 cultures) were used in all the experiments. Every 24 h, the medium temperature and pH (Jenway pH Meter 3310) were measured. Culture growth was determined by the cell density of samples collected every 24 h, by counting cells using a Neubauer improved bright-line chamber and a microscope (Leica DM 2000). At the end of the bioassay (96 h) the endpoints, average specific growth rates per day [μ (day 0–4) [d^{-1}]] and the inhibition of average specific growth rate were calculated from cell density measurements.

2.3.2. Combined effect of paraquat and lysine acetylsalicylate on C. vulgaris growth

The experiments with the mixture of PQ and LAS were divided into two different assays. PQ EC75 value (1.38 mg/L) determined in the toxicity test previously described was used for the mixture assays. LAS was mixed with PQ at different concentrations [0.5 (2.18 mg/L), 1 (4.36 mg/L) and two times (8.72 mg/L) the molar concentration of PQ]. In the other assays, PQ EC50 (0.5 mg/L) was used for the mixture assays with higher concentrations of LAS that correspond to molar proportions of 2 (2.42 mg/L), 4 (4.82 mg/L), 8 (9.65 mg/L), 16 (19.3 mg/L) and 32 times (38.6 mg/L):

(a) Each flask of CM (400 mL) containing *C. vulgaris* was divided in the following groups: (i) control group, (ii) PQ EC75 group, (iii) PQ + LAS 1:0.5 group, (iv) PQ + LAS 1:1 group, (v) PQ + LAS 1:2 group (vi) LAS 0.5 group, (vii) LAS 1 group, (viii) LAS 2 group;
(b) Evaluation of the toxicity of higher concentrations of LAS. The concentration of LAS tested corresponded to the higher concentration used (38.6 mg/L). Each flask of CM (400 mL) containing *C. vulgaris* was divided in the following groups: (i) control group, (ii) LAS 32, (iii) PQ EC50 group, (iv) PQ + LAS 1:2 group, (v) PQ + LAS 1:4 group, (vi) PQ + LAS 1:8 group, (vii) PQ + LAS 1:16 group, (viii) PQ + LAS 1:32.

2.4. Stability and biotransformation experiments

For the evaluation of the stability of the compounds, each flask of 400 mL containing only culture medium was divided in the following groups: LAS 32 (38.6 mg/L and PQ + LAS 1:32 (0.5 mg/L + 38.6 mg/L). The same solutions were added to the culture medium in the presence of *C. vulgaris* for the evaluation of putative biotransformation. Both assays were undertaken according with the described above for the first experiment. All the parameters determined for the toxicity test at 96 h were also performed.

2.4.1. Lysine acetylsalicylate, salicylic acid and paraquat quantification in filtrate and algal cells

After 96 h of exposure to the compounds in the biotransformation experiment, an aliquot of 0.5 mL from PQ, LAS 32 and PQ + LAS 1:32 groups were centrifuged (4000*g*, 20 min, 4 °C) and the supernatant recovered and stored (−20 °C) for further analysis. The pellet was reconstituted in HCl 0.01 M and stored (−20 °C) until analysis. Before analysis, both solutions were filtered (0.45 μm Milipore®). LAS and SA were quantified by a previously reported high-performance liquid chromatography (HPLC) with UV detection method (Kees et al., 1996). Standards of LAS and SA were prepared in HCl 0.01 M and the internal standard, 4-hydroxybenzoic acid, was added to an aliquot of the standards, supernatant and reconstituted pellet at the final concentration of 20 μg/mL and 50 μL of the sample was then injected in a HPLC system consisting of a pump (Gilson model 302; Gilson Medical Electronics, Villiers le Bel, France) connected with a manometric model (Gilson, model 802 C) and Spherisorb S10 ODS2 Column (Oasis®) of 25 cm × 4.6 mm. An autosampling injector (Gilson, model 231–401) was used to make the injections into the HPLC system by means of a 20 μL loop. The detection was carried out on ultraviolet detector (Gilson UV/VIS-155) at 237 nm (Kees et al., 1996). The mobile phase was a solution of water:acetonitrile:orthophosphoric acid (85%) (80:19.9:0,1), pH 2.5, filtered through a 0.45 μm filter (Milipore, Avenida del Liano Castellano, 13-28034 Madrid, Spain) and degassed with nitrogen gas. The flow rate was 1 mL/min. Data collection and processing were carried out using Clarity software®, basic edition, clarity version 2.6.6.574.

PQ extraction from the supernatant and cell lysate was performed according to (Moreira et al., 2012). Briefly, an aliquot of 0.5 mL of each aqueous supernatant, 1.5 mL of phosphate buffered saline solution (pH 8.0) and 20 μL of the internal standard, ethyl paraquat (EPQ) solution (100 μg/mL) were pipetted into a 15 mL plastic tubes. Ten milligrams of $NaBH_4$ were added to the mixture in order to reduce PQ and the EPQ to the hydrogenated derivatives accordingly to (Draffan et al., 1977). The reduction was left to occur at 60 °C for 10 min and the mixture was then kept in cold water for 2 min. Solid phase extraction (SPE) was performed using Bond Elut® C18 cartridges installed on a vacuum manifold. The cartridges were preconditioned with 2 mL of methanol and 2 mL phosphate buffer (pH 8). The sample was transferred to the SPE column, which was washed with 2 mL of deionized water. Afterwards, the samples were eluted under vacuum, at a flow/rate of 1.5 mL/min with 2 mL of methanol. The eluate was evaporated at room temperature under a gentle stream of nitrogen. The residue was reconstituted in 100 μL of methanol and 1 μL was injected in the GC-IT/MS system (Moreira et al., 2012).

2.5. Data and statistical analysis

One-way ANOVA followed by a Dunnett" test for multiple comparisons was employed to ascertain if growth rates determined to each treatment were significantly different from those of the control (Zar, 1996). No observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) values were taken from ANOVA significant results, reported for $p < 0.05$.

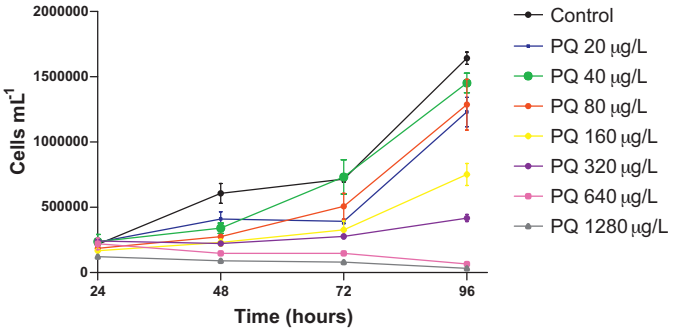

Fig. 1. Growth curves of *Chlorella vulgaris* exposed to different concentrations of paraquat (PQ). Values are given as mean ± S.D.

The average specific growth rate per day was calculated as the logarithmic increase in biomass in each test from the equation (OEDC, 2006):

$$\mu_{i-j} = \frac{I_n B_j - I_n B_i}{t_j - t_i}$$

where μ_{i-j} is the average specific growth rate from time i to j, B_i is the biomass at time i and B_j is the biomass at time j.

For each treatment, the percent inhibition of growth rate was calculated for each treatment replicate using the equation (OEDC, 2006):

$$\%I = \mu_c - \mu_t$$

where $\%I$ is the percent inhibition in average specific growth rate, μ_c and μ_t are the mean value for average specific growth rates (μ) of the control and treatment, respectively. The $\%I$ values calculated for each experiment were transformed by probit analysis and plotted against the logarithm of the test substance concentration to obtain the toxicity curves. From each curve, the 20%, 50% and 75% inhibition concentrations (EC_{20}, EC_{50} and EC_{75} respectively) were calculated.

Results are presented as means ± standard deviation and statistical comparisons between groups were performed with one-way ANOVA (in case of normal distribution) or Kruskal–Wallis test (one-way ANOVA on ranks–in case of distribution is not normal). Statistical calculations were performed with the GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego CA, USA) and SPSS 17.0 software package for Probit analyses. In all cases, p values lower than 0.05 were considered as statistically significant.

3. Results

3.1. Growth inhibition test

Growth rate of microalgae cultures exposed to PQ was significantly reduced after 48 h of exposure, at concentrations above 40 μg/L ($p < 0.05$). For concentrations higher than 160 μg/L the growth rate was even more significantly reduced ($p < 0.001$) and this growth pattern was maintained until 96 h of exposure (Fig. 1). After 96 h of exposure to the higher PQ concentration, *C. vulgaris* growth was almost completely inhibited, showing a growth rate of 0.296 day^{-1}, near 25% of control cultures growth rate. The EC_{20}, EC_{50} and EC_{75} values calculated from the toxicity curves (log concentration vs. probit transformation of $\%I$ values), at the end of 96 h, are shown in Table 1. The toxicity curve (log of PQ concentration vs.% of growth inhibition) at 96 h obtained for different PQ concentrations is shown in Fig. 2.

Table 1
Estimated no observed effect concentration (NOEC), lowest observed effect concentration (LOEC), and concentrations of paraquat (PQ) inducing 20%, 50% and 75% (EC_{20}, EC_{50} and EC_{75}, respectively) inhibition of *Chlorella vulgaris* population growth after 96 h of exposure. 95% confidence intervals are indicated within brackets.

	NOEC	LOEC	$EC_{20(95\%CI)}$	$EC_{50(95\%CI)}$	$EC_{75(95\%CI)}$	Pearson' correlation coefficient
PQ (μg/L)	80	160	$195.951_{(162.197–230.111)}$	$525.483_{(452.687–620.434)}$	$1158.498_{(944,580–1498,678)}$	0.982

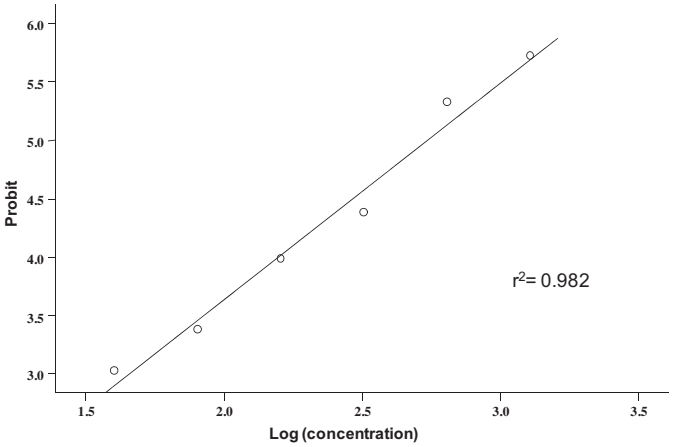

Fig. 2. *Chlorella vulgaris* growth inhibition probit curve as a function of log concentration of paraquat (PQ). The r^2 obtained is also indicated.

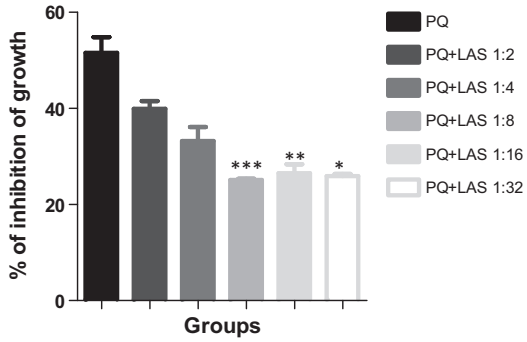

Fig. 4. *Chlorella vulgaris* population growth inhibition after 96 h of exposure to paraquat (PQ). EC_{50} (0.5 mg/L). Increasing concentrations of lysine acetylsalicylate (LAS) were added to the formulation, corresponding to 1:2; 1:4; 1:8; 1:16; 1:32 of PQ + LAS in molarity. Values are given as mean ± S.D. * *versus* PQ ($p < 0.05$); ** $p < 0.01$; *** $p < 0.001$.

When LAS was added to the PQ formulation, the toxicity profile was maintained. Noteworthy, despite the lack of differences between the growth rates of PQ and PQ + LAS (Fig. 3A), the % of inhibition of growth was reduced in both groups PQ + LAS 1:1 and PQ + LAS 1:2 (Fig. 3C). On the other hand, *C. vulgaris* growth was stimulated by LAS as shown in Fig. 3B. In subsequent experiments, LAS was added at higher concentrations to evaluate optimal protective concentrations. From the proportion of 1:8, LAS conferred a protection against PQ toxicity of approximately 1.8 fold as observed by the reduction in the % of inhibition of growth (Fig. 4).

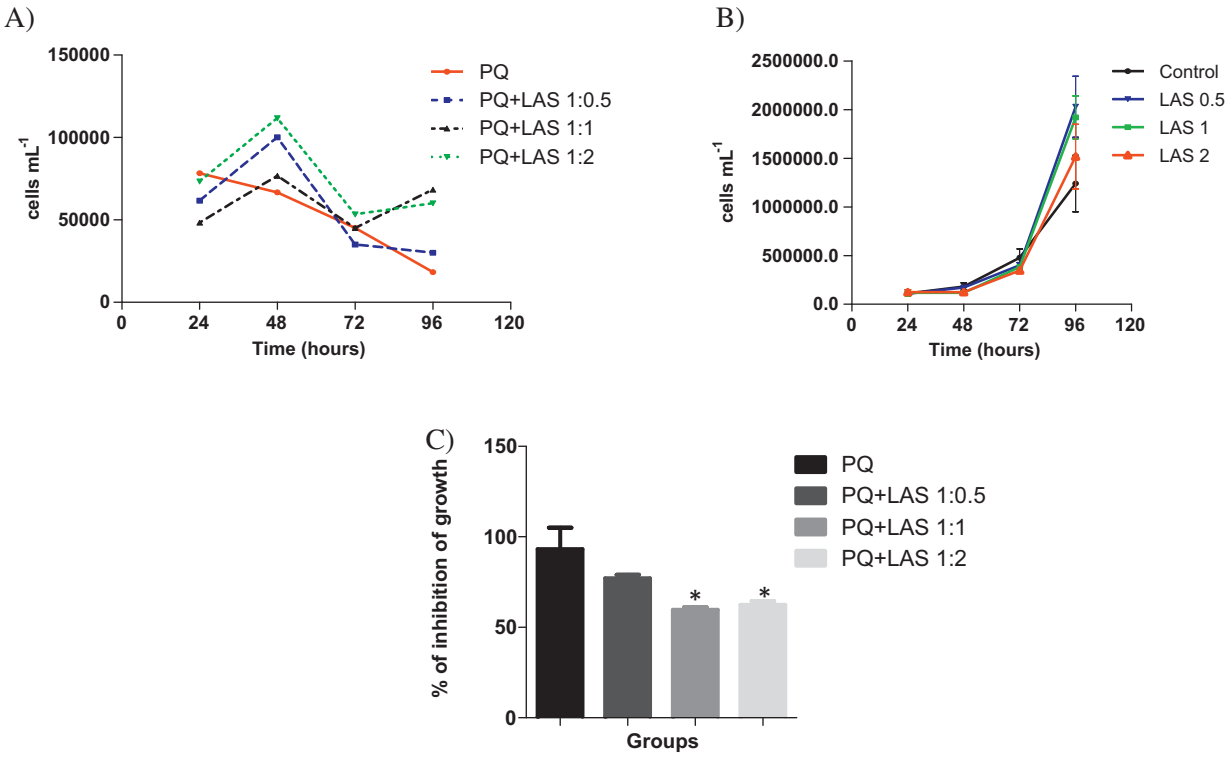

Fig. 3. A) *Chlorella vulgaris* population growth curves exposed to paraquat (PQ) IC_{75} (1.38 mg/L), three concentrations of lysine acetylsalicylate (LAS) were added to the formulation, corresponding to 1:0.5, 1:1 and 1:2 (PQ + LAS) in molarity. Standard deviations were omitted for better visualization. B) Growth curves of *Chlorella vulgaris* exposed only to LAS. C)% of inhibition of growth of *Chlorella vulgaris* exposed to PQ IC_{75}. Increasing concentrations of LAS were added to the formulation, corresponding to 1:0.5; 1:1; 1:2 of PQ + LAS in molarity. Values are given as mean ± S.D. * *versus* PQ ($p < 0.05$).

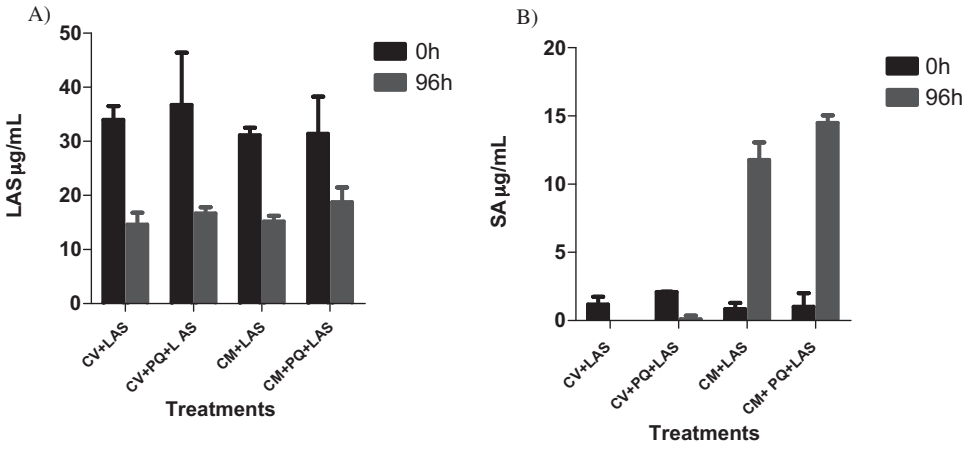

Fig. 5. Concentrations of lysine acetylsalicylate (LAS) (5A) and its metabolite salicylic acid (SA) (5B) in the extracellular media of the groups LAS 32 and PQ + LAS 1:32, in the presence or absence of *Chlorella vulgaris*. Aliquots of 0.5 mL were taken at 0 h and at 96 h; cells were removed by centrifugation before analysis by HPLC. CV: *Chlorella vulgaris*; CM: culture media. Values are given as mean ± S.D.

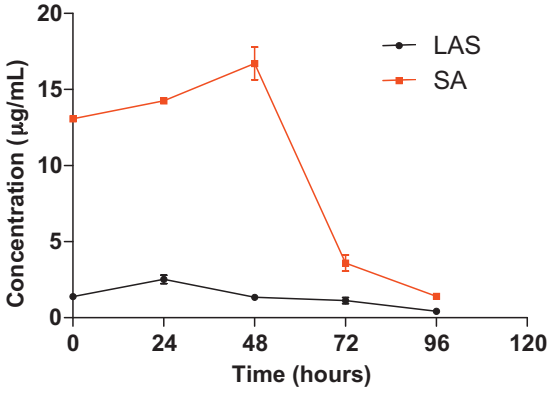

Fig. 6. Time course of the biotransformation of lysine acetylsalicylate (LAS) and salicylic acid (SA) biodegradation by *Chlorella vulgaris*. Aliquots of 0.5 mL were taken daily from incubations for four days; cells were removed by centrifugation before analysis by HPLC-UV. Values are given as mean ± S.D.

3.2. Stability and biotransformation experiments

Regarding the stability of LAS in the culture medium, analysis by HPLC of cell-free supernatants, at the end of the experiment (96 h), showed that approximately 50% of the added LAS was depleted (Fig. 5A). The depletion of LAS was mainly due to its spontaneous hydrolysis into SA in aqueous medium, as shown in Fig. 5B Surprisingly, when LAS was added to the medium containing *C. vulgaris*, SA was almost undetectable at the end of 96 h, being only quantified in the first minutes after exposure (Fig. 5B). Consequently, to understand the *C. vulgaris*-mediated biotransformation of LAS, the quantification of LAS and SA was performed daily in the supernatant and in the cellular pellet. As demonstrated in Fig. 6, the peak of SA concentration is achieved after 48 h of exposure and SA abruptly disappears afterwards. The time course of LAS biodegradation is also shown in Fig. 6. In contrast, PQ was stable throughout the experiment, whether *C. vulgaris* was present or not in the culture medium (data not shown).

4. Discussion and conclusion

The present work was undertaken to assess if the new formulation of PQ including LAS had a lower toxicity than the commercially available (Gramoxone®) to freshwater primary producers using *C. vulgaris* as test organism. Our results clearly demonstrated that LAS is able to protect green algae from PQ-induced toxicity. During application of PQ, non-target species can be affected through the dispersion of the herbicide in the environment, by entering into aquatic ecosystems from agricultural runoff or leaching, among other processes. PQ is toxic to a wide variety of microorganisms (Carr et al., 1986), especially to primary producers, such as microalgae (Ibrahim, 1990; Saenz et al., 1997). The toxicological mechanisms of PQ in photosynthetic organisms and bacteria at the biochemical, physiological and molecular level are well understood (Carr et al., 1986; Diaz et al., 1980; Ekmekci and Terzioglu, 2005; Smith, 1988). In plants and algae, PQ^{2+} inhibits photosynthesis by accepting electrons from photosystem I, leading to the generation of $PQ^{\cdot+}$, which initiates a series of oxidative reactions, leading to cell membrane disruption and plant death (Dinis-Oliveira et al., 2008b; Slade, 1966; Zweig et al., 1965). The deviation of electron flow to $NADP^+$ inhibits its reduction to NADPH, thus compromising the normal functioning of photosystem I. In the presence of O_2, $PQ^{\cdot+}$ is reoxidized to PQ^{2+} and one electron is transferred to O_2 forming superoxide radicals and leading to the generation of other reactive species such hydroxyl and peroxyl radicals (Slade, 1966). Additionally, at the molecular level, PQ inhibits the *psa*B and the *rbc*L gene that codes for the photosystem I reaction center protein and for the large subunit of ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco), respectively (Qian et al., 2009). After only 24 h of herbicide exposure, significant DNA damage was observed in microalgal cells, with a 23.67% of comets in cultures exposed to 0.05 µM, revealing the genotoxicity of this herbicide (Prado et al., 2009).

Previous toxicity tests of PQ in microalgae have been performed in several species with a wide difference in sensitivity (Ibrahim, 1990; Jamers and De Coen, 2010). Saenz and colleagues, found that EC50 of PQ in *C. vulgaris*, following exposure for 96 h, was 140 µg/L and for *Scenesdesmus* species ranged from 47 to 670 µg/L (Saenz et al., 1997). Ma et al. assessed the toxicity of 40 herbicides to *Raphidocelis subcapitata* and *C. vulgaris* and their results showed that *C. vulgaris* was more sensitive to PQ than *R. subcapitata* (0.2 µg/L *versus* 18.7 µg/L, respectively) (Ma et al., 2002, 2006). Comparatively, under our laboratory conditions, *C. vulgaris* was more resistant to PQ, since the EC_{50} calculated was 525 µg/L (Table 1), four times higher than the EC_{50} previously described for *C. vulgaris* (Ibrahim, 1990; Jamers and De Coen, 2010; Ma et al., 2002, 2006; Saenz et al., 1997).

Most of the previous studies on PQ toxicity to microalgae use biomass, growth rate and its inhibition as endpoints. In accordance, in our experimental model we evaluated the inhibition of *C. vulgaris*

population growth in the presence of Gramoxone® and the new formulation PQ + LAS. For proportions of PQ + LAS 1:2 (mol/mol) or higher it was already possible to observe a slight protection, shown by a decrease of the % of growth inhibition (Fig. 3C and Fig. 4). LAS conferred a protection against PQ toxicity of approximately 1.8 fold when LAS was added in concentrations above 9.65 mg/L (Fig. 4).

In mammals, LAS and SA possess a well-known antioxidant and anti-inflammatory activity with remarkable hydroxyl scavenging properties, inhibition of the transcription factors NF-κB and AP-1 and inhibition of gene expression of COX-2, iNOS and IL-4 (Baltazar et al., 2011). In the present experiment LAS was used as a fundamental component of a new herbicide formulation recently developed by our group (Baltazar et al., 2013a). LAS is the water-soluble lysine salt of acetylsalicylic acid, which is rapidly deacetylated to SA in aqueous solutions at pH values above 7. At the beginning of the experiment, SA was already detected, suggesting that the degradation of LAS occurred rapidly in the culture medium. As shown in Fig. 6, after 48 h almost all LAS has been hydrolyzed to SA. It was also observed that SA is, to some extent, biotransformed by *C. vulgaris* after 48 h (Fig. 6), not being detected after 96 h in the culture media (Fig. 5B). The rapid removal of SA by *C. vulgaris* is in accordance with other recent studies which suggest that microalgae, and mainly *Chlorella* species, play an important role in the bioremoval and biotransformation of a variety of toxic organic pollutants such as bisphenol (Hirooka et al., 2005), nonylphenol (Gao et al., 2011), tributylin (Tam et al., 2002), estrogenic contaminants (Della Greca et al., 2008), pyrene (Lei et al., 2002), and metals (Khoshmanesh et al., 1996; Nacorda et al., 2010; Wilde and Benemann, 1993). Due to the rapid uptake of SA, it seems that the protection against PQ toxicity might be due to the release of SA in the aquatic medium resultant from the hydrolysis of LAS. In fact, SA is a well-known plant natural signaling molecule for the activation of defense-related genes in response to pathogen infection and stress factors (Klessig and Malamy, 1994). Pre-treatment of seedlings with 0.5 mM SA for 24 h before exposure to 10 μM PQ and light reduces PQ-induced chlorophyll losses, H_2O_2 production, lipid peroxidation, electrolyte leakage, and completely blocked the inhibitory effect of the herbicide on photosynthesis (Ananieva et al., 2004). Czerpak and colleagues have shown that SA stimulates algal growth, protein synthesis and the expression of nucleic acids, which partially might explain the higher algal growth in the groups treated with LAS versus the control group as shown in Fig. 3B (Czerpak et al., 2002). SA also increases the intensity of photosynthesis in algae, possibly due to an increase of the photosynthetic pigments (chlorophyll *a*, *b* and total carotenoids) synthesis (Czerpak et al., 2002). One of the major ways in which plants regulate their defense mechanisms in response to abiotic and biotic stresses is the ROS sensing, producing and scavenging system (Foyer and Noctor, 2013). SA exogenously added to barley plants prevented PQ induced-toxicity by enhancing the activity of antioxidant enzymes (Ananieva et al., 2004). SA-deficient NahG transgenic rice plants showed a reduction of the glutathione pool and consequently, a greater susceptibility to oxidative damage induced by PQ (Kusumi et al., 2006; Yang et al., 2004). High levels of necrotrophic pathogen-induced SA corresponded to high levels of glutathione, supporting the concept that in plants there is a tight correlation between both molecules (Kuzniak et al., 2013).

Although the studies mentioned above support the theory that SA protection against PQ toxicity might be related to the antioxidant capacity of these drugs (Ananieva et al., 2004; Baltazar et al., 2011; Kawano and Muto, 2000), another mechanism might be involved. Dinis-Oliveira and colleagues proved that SA and PQ react in aqueous medium forming stable charge-transfer complexes (Dinis-Oliveira et al., 2008a), which in theory prevents PQ from undergoing the redox cycling, thus limiting its toxicity. As mentioned before, in our experimental model, SA is rapidly released from LAS hydrolysis in the aqueous medium (Fig. 5B and Fig. 6), thus available to complex with PQ. Silverman and colleagues have shown that the simultaneous foliar application of PQ with sodium salicylate significantly reduced the PQ-induced toxicity (Silverman et al., 2005). The authors concluded that the SA-mediated protection was independent from systemic acquired resistance (SAR) induction, ethylene, and alternative respiration, but rather related to interferences with PQ uptake, stimulation of antioxidant defenses, and direct scavenging of radical species (Silverman et al., 2005). Once again, despite the important contribution of SA antioxidant activity, the reactivity of PQ with SA should also be considered. Although higher plants have the same target sites in photosynthesis as *Chlorella*, our group recently demonstrated that PQ + LAS in the proportion of 1:2 maintained the herbicidal effect (Baltazar et al., 2013a). In our previous studies, the herbicidal activity of the formulation with six-month-old was weaker at the end of the experiment by comparison with the efficacy of the freshly prepared solution. This partial loss of activity could be explained by the hydrolysis of LAS into SA with time within the solution. In order to avoid the interference of SA with the herbicidal effect of PQ, the formulation has to be applied immediately after preparation to overcome the instability problem with LAS. At the same time, the hydrolysis of LAS in the aquatic medium is advantageous to the environment as seen by the stimulation of algal growth in the presence of SA.

In conclusion, our work demonstrated that the new formulation with PQ + LAS has a reduced toxicity to *C. vulgaris*, suggesting a lower risk of its use to freshwater primary producers relatively to other PQ formulations. Together, it seems that SA metabolism, antioxidant capacity and modulation of oxidative stress contributes to SA-signalling and perception pathways of cytoprotection against PQ toxicity. However, in order to further investigate the environmental effects of this new formulation, similar studies using other aquatic organisms, different trophic levels of freshwater ecosystems and soil persistency or biodegradation studies should be conducted.

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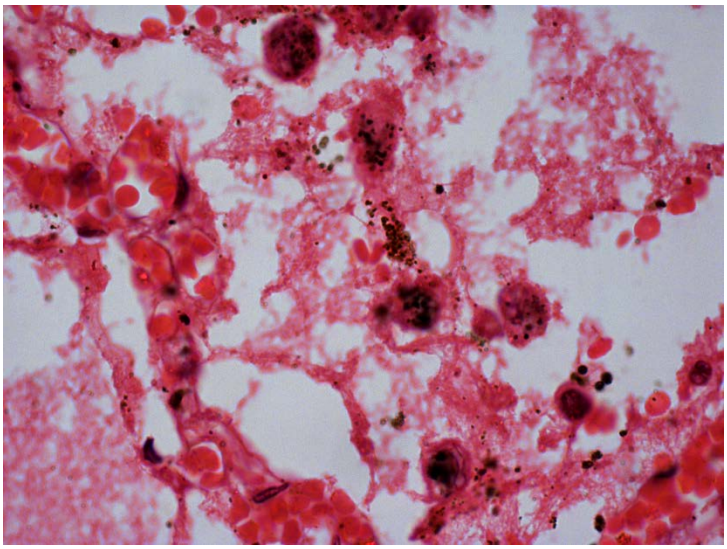
Study III

Lysine acetylsalicylate improves the safety of paraquat formulation in rats, by increasing its elimination, and preventing lung and kidney injury

(Submitted for publication)



Lysine acetylsalicylate improves the safety of paraquat formulation in rats, by increasing its elimination, and preventing lung and kidney injury

Running title: Lysine acetylsalicylate improves the safety of paraquat

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Abstract

The incorporation of lysine acetylsalicylate (LAS) in the commercial formulation of paraquat (PQ), Gramoxone®, has been shown to significantly increase the survival of intoxicated mammals, while maintaining the herbicidal effect. The aim of the present study was to clarify the mechanisms involved in the protective effect of LAS in a rodent model, through monitoring PQ levels and the histological and biochemical biomarkers of toxicity in male Wistar rats following intoxication with PQ formulations in the absence and presence of LAS. Gramoxone® and the formulation with LAS were administered by gavage, at the doses of 125 mg/kg of PQ and 125 mg/kg of PQ + 316 mg/kg of LAS, respectively. The obtained results showed that LAS improves the safety of PQ formulation, by increasing its elimination, and preventing lung and kidney injury. LAS prevented the biochemical and histological alterations in lung induced by PQ at the end of 24h and 48h. This was evidenced by a significant reduction in lipid peroxidation, the maintenance of reduced glutathione levels and decreased levels of oxidized glutathione, as well as the normalization of the urinary biomarkers, creatinine and *N*-acetyl- *β*- glucosaminidase. LAS treatment also caused a significant reduction in PQ-induced activation of nuclear factor kappa B (NF-κB) in lung. The results allow us to conclude that lysine acetylsalicylate improves the safety of PQ formulation in rats, by increasing its elimination, and preventing lung and kidney injury.

Keywords: Paraquat, antidote, lysine acetylsalicylate, herbicide.

Introduction

The human fatality rate resulting from intoxications with the herbicide paraquat (1,10-dimethyl-4,40-bipyridinium chloride; PQ) is very high due to both its inherent toxicity and the lack of effective treatments. As PQ is still widely used in numerous countries, several measures to prevent poisoning or reduce its toxicity have been implemented since its introduction in the market (Baltazar et al., 2013a; Dinis-Oliveira et al., 2009a; Dinis-Oliveira et al., 2009b; Dinis-Oliveira et al., 2007a; Dinis-Oliveira et al., 2007b). Initially, risk mitigation measures were applied in the PQ formulation, as the addition of a blue-green dye, stenching agents, thickeners, reduction of the concentration of PQ (10%-20%), improvement of the safety caps and labeling (Sabapathy, 1995). More recently, a new formulation (INTEON®) was developed to prevent the absorption of PQ and increase its elimination through the incorporation of an emetic, an alginate to thicken the formulation in the acidic environment of the stomach, and an osmotic purgative to speed its elimination from the small intestine (Heylings et al., 2007; Wilks et al., 2008). However, subsequent surveys were unable to link the improvement of the survival rate with the new formulation (Wilks et al., 2011).

In fact, PQ poisonings are still associated with a mortality rate of 40 to 60%, and most of the fatal cases occur within 24–72 h, due to multi-organ failure (Gil et al., 2008; Senarathna et al., 2009). PQ toxicity is especially severe in the lungs due to its accumulation, against a

concentration gradient, through the highly developed polyamine uptake system, and due to its capacity to continuously generate reactive oxygen species (ROS) through a redox cycle involving PQ radicals and oxygen (Smith, 1982, 1987). The development of pulmonary lesions is characterized by an early destructive phase (1-3 days) with an acute alveolitis and extensive influx of inflammatory cells; and a delayed proliferative phase (3-7 days) that involves the development of extensive fibrosis in the lung, which results in the loss of the normal alveolar architecture, interfering with gaseous exchange, and subsequently causing death from anoxia.

Taking into account the mechanism of PQ toxicity, involving a strong inflammatory reaction and ROS production, it was postulated that an antidote against PQ poisoning should have excellent antioxidant and anti-inflammatory properties, involving the inhibition of nuclear factor κB (NF-κB), AP-1 and COX-1 activation (Gawarammana and Buckley, 2011). Nevertheless, in spite of anecdotal successful clinical cases (Dinis-Oliveira et al., 2006c; Jenq et al., 2005; Lee et al., 2009), the efficacy of the decontamination with fuller´s earth, and with the anti-inflammatory and immunosuppressive therapies is still very poor (Dinis-Oliveira et al., 2009a; Dinis-Oliveira et al., 2006c; Suntres, 2002; Suntres and Shek, 1995; Yeh et al., 2006).

Recently, salicylates have emerged as effective antidotal candidates to overcome the lack of an effective treatment due to their well-known anti-inflammatory and antioxidant action (Baltazar et al., 2011). Animals exposed to a toxic dose of PQ (25 mg/kg, ip) and treated 2h after with sodium salicylate (NaSAL) (200 mg/kg ip) had a significant reduction in PQ-induced oxidative stress, platelet activation, and NF-κB activation in lung (Dinis-Oliveira et al., 2007b). NaSAL also prevented the PQ induction of apoptosis in rat lung and all of these changes were accompanied by the full survival of the animals (Dinis-Oliveira et al., 2007a; Dinis-Oliveira et al., 2007b). Additionally, further research was performed with the salicylate prodrug, lysine acetylsalicylate (LAS) to overcome the problem with solubility for future intravenous and intramuscular administrations in hospitals, and similar efficacy was obtained(Dinis-Oliveira et al., 2009b).

Our most recent approach to decrease the risk liability of PQ was to develop a new formulation of PQ containing the antidote LAS with decreased mammalian toxicity while maintaining effective herbicidal activity (Baltazar et al., 2013a). The PQ solution used to develop the formulation was the commercialized by Syngenta Ldt., Gramoxone (20% w/v) to ensure a realistic approach to this study. The survival rate of rats orally exposed to 125 mg/kg of PQ ion was only 40%, while the presence of LAS in the formulation (125 mg/kg of PQ ion and 316 mg/kg of LAS) provided effective protection, as confirmed by the full survival observed. Noteworthy, both formulations of PQ, either in the absence or in the presence of LAS, provided the same herbicidal activity (Baltazar et al., 2013a).

In the present work, we evaluated the acute oral toxicity of this new formulation following an up-and-down protocol (UDP), recommended by the Organisation for Economic Co-operation and Development (OECD), in alternative to the conventional

LD50 testing. Additionally, it was also our aim to provide comprehensive PQ measurements, as well as lung histological evaluation and biochemical data in plasma and urine that could help to clarify the mechanisms involved in the protective effect of LAS, which led to full survival in the previous study (Baltazar et al., 2013a).

Material and Methods

Chemicals and drugs

The commercial formulation of PQ used was Gramoxone® [20% PQ (1,1´-dimethyl-4,4´-bipyridinium dichloride hydrate; molecular mass=257.2 g/mol], kindly supplied by Syngenta Crop Protection Lda (Lisboa, Portugal). Ampoules of LAS (molecular mass = 326.35 g/mol) and water for injections were a generous gift from Labesfal Genéricos (Campo de Besteiros, Portugal). The formulation of LAS corresponds to the same available for human treatments in Portugal. The reagents for the albumin, total protein, total bilirubin, direct bilirubin, cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides (TG), glucose, creatinine, urea, aspartate aminotransferase (AST), alanine aminotransferase (ALT), amylase, creatinine kinase (CK), creatinine kinase MB (CK-MB), lactate dehydrogenase (LDH), lactate, uric acid, α-1-antitrypsin, potassium, sodium, alkaline phosphatase (ALP), γ-glutamyl-transpeptidase (GGT), C-reactive protein (CRP) were obtained from PZ Cormay S.A. *N*-acetyl- *β*- glucosaminidase (NAG) reagents were obtained from Diazyme Europe GmbH.

Animals

A total of 57 adult male Wistar rats (aged 8 weeks) obtained from Charles River S.A. (Barcelona, Spain), with a mean body weight of 269 ± 37g, were used. Animals were kept in standard laboratory conditions (12/12 h light/darkness, 22±2ºC room temperature, 50-60% humidity) for at least 1 week before starting the experiments. Animals were allowed access to tap water and rat chow ad libitum during the quarantine period. Housing and experimental treatment of the animals were in accordance with the Guide for the Care and Use of Laboratory Animals from the Institute for Laboratory Research. The experiments complied with current Portuguese laws.

Determination of the acute oral toxicity by the up-and-down (UDP) procedure

A total of 17 adult female Wistar rats (aged between 8-12 weeks) obtained from Charles River S.A. (Barcelona, Spain), with a mean body weight of 199 ± 15 g, were used. Doses were administered by gavage, in a standard volume of 0.5 mL according to the UDP schedule, and were made by diluting Gramoxone® in water or mixing Gramoxone® with LAS in the proportion 1:2. OECD Guideline 425 was followed to estimate the acute oral toxicity (LD50) value along with its confidence interval (OECD, 2008). The main test of this guideline was used. Briefly, it consists of a single-ordered dose progression in which animals are dosed individually (one at a time) and observed, for 48h, before a subsequent dose is administered to another animal. The first animal receives a dose a step below the level of the best estimate of the LD50. For PQ, the oral LD50 in male

Wistar rats described is 125 mg/kg (Dinis-Oliveira et al., 2009b; Kimbrough and Gaines, 1970; Murray and Gibson, 1972) and for that reason the first animal received the dose below in the progression, 40 mg/kg. The dose progression for each subsequent animal was determined by the short-term (48 h) fate of the previously dosed one. If an animal survived within the short term interval, the next animal received a higher dose. If this last animal died within this time period, the dose progression proceeded with a lower dose (see Tables 1 and 2) as prescribed in the Acute Oral Toxicity (AOT) software program (OECD, France) used for the analysis of dosing data. The animal's long-term fate at the end of 14 days after exposure was also recorded. Dosing continued until one of the three standard stopping criteria was met: i) three consecutive animals survived at the upper bound of dosing, ii) five reversals occurred in any six consecutive animals tested (when a reversal is created by a pair of responses in a situation in which a nonresponse is observed at a particular dose and a response is observed at the next dose tested, or vice versa), or iii) at least four animals have followed the first reversal and the specific likelihood ratios (see (OECD, 2008)) exceed the critical value. All animals were observed individually for toxicological signs, especially in the first hours post-dosing and until the end of experiment (14 days). Body weight, locomotor activity, dyspnea, nasal hemorrhage, skin and fur characteristics were recorded if present. After the stopping criteria had been reached, an estimate of the LD50 value and the associated confidence limits were calculated using the AOT software (Guideline 425) Statistical Program version1.0.

Acute toxicity at the end points 24h and 48h

Experimental protocol

After the quarantine period, 40 animals were randomly divided into eight groups of five animals each. Each animal was individually housed in a metabolic cage where it was kept during the whole time of experiment (24h and 48h). Animals were fasted for 24h before sacrifice, but water was given *ad libitum* during the whole experiment. Urine and feces were collected over ice during the 24h or 48h period, for quantification of PQ and biochemical parameters. The PQ administered dose (125 mg/kg of PQ ion) corresponds to the oral LD_{50} reported by other authors (Dinis-Oliveira et al., 2009b; Kimbrough and Gaines, 1970; Murray and Gibson, 1972). Based on our previous results showing full survival, the proportion of PQ and LAS used in the experiments was 1:2 in molarity (Baltazar et al., 2013a). The aqueous administrations of vehicle (distilled water), PQ and the mixture PQ+LAS were all done by gavage in a volume of 0.5 mL/250 g of body weight.

Each group of animals was treated as follows: (i) Control group, n=10: animals were treated with distilled water and sacrificed at 24 h (n=5, control 24 h), and 48 h (n=5, control 48 h). (ii) LAS group, n=10: animals were treated with LAS (316 mg/kg) and sacrificed at 24 h (n=5, LAS 24 h), and 48 h (n=5, LAS 48 h). (iii) PQ group, n=10: animals were treated with Gramoxone® (125 mg/kg PQ ion) and sacrificed at 24 h (n=5, PQ 24 h), and 48 h (n=5, PQ 48 h). (iv) PQ+LAS group n=10: animals treated with PQ (125 mg/kg) + LAS (316 mg/kg) and sacrificed 24 h (n=5, PQ+LAS 24 h), 48 h (n=5, PQ+LAS 48 h).

Sample collection and processing of samples

Twenty four and fourty eight hours after administrations, anesthesia was induced with xylazine/ketamine (10 mg/kg/100 mg/kg i.p). Animals were placed in the *decubito supino* position and the thorax was opened by two lateral transversal incisions and one central longitudinal incision to expose the aorta artery. Blood was collected with a heparinized needle from the aorta artery into a heparinized containing tube. Lungs were removed, cleaned of all major cartilaginous tissues of the conducting airways, weighed, and processed as follows: the left lung was homogenized (1:4 m/v) with an Ultra-Turrax® homogenizer in cold mixture of phosphate buffer [(1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, 8 g NaCl e 0.2 g KCl), pH 7.4] and centrifuged (3000g, 4°C, for 10 min). Aliquots of the resulting supernatants were transferred to eppendorfs and centrifuged again (13000 *g*, 4ºC, for 10 min). The resulting supernatants were divided in aliquots and stored (−80°C) until posterior quantification of PQ, mieloperoxidase (MPO) activity, and protein content. Perchloric acid (10%) was added to the resulting supernatant described above in the proportion of 1:1 and then centrifuged at 13000 *g*, 4ºC, for 10 min. The resulting supernatant was stored (−80°C) until quantification of total glutathione (GSHt), reduced glutathione (GSH), oxidized glutathione (GSSG) and lipid peroxidation. The right lung was prepared for histological and immunohistochemical analysis, as following described.

Blood samples were centrifuged (3000g, 4°C, for 10 min) and plasma was aliquoted and stored (−80°C) for further biochemical analysis (albumin, total protein, total bilirubin, direct bilirubin, cholesterol, HDL, LDL, TG, glucose, creatinine, urea, AST, ALT, amylase, CK, CK-MB, LDH, lactate, uric acid, α-1-antitrypsin, potassium, sodium, ALP, GGT, CRP) and PQ quantification. Feces were homogenized in distilled water (1:4 m/v) and were kept in constant shaking overnight, then centrifuged (3000g, 4°C, for 10 min). The supernatants were stored (−80°C) until quantification of PQ. Urine samples were centrifuged (3000g, 4°C, for 10 min) and stored (−80°C) until quantification of PQ, urea, creatinine and NAG. Clearance of creatinine was calculated as follows:

$$\text{Clearance of creatinine (mL/min/kg)} = \frac{\text{Urinary creatinine} \times \text{urine volume 24h}}{\text{Plasmatic creatinine} \times 1440 \times \text{body weight}}$$

Biochemical analysis

Plasma biochemical parameters were measured in duplicate on an AutoAnalyzer (PRESTIGE 24i, PZ Cormay S.A.). Urinary urea, creatinine and total proteins were measured in duplicate according to formerly described methods (Fabiny and Ertingshausen, 1971; Talke and Schubert, 1965). Urinary NAG activity was assayed in duplicate as previously reported (Yuena C.T, 1982). One Unit of NAG was defined as the amount of enzyme that releases one µmol of p-nitrophenol in the assay conditions. Results were expressed in U/L.

Tissue protein was quantified in triplicate according to the method of (Lowry et al., 1951), using bovine serum albumin as standard. Lung lipid peroxidation was evaluated in triplicate by the thiobarbituric acid reactive substances methodology (Buege and Aust, 1978). Results were expressed as nmol

of malondialdehyde (MDA) equivalents/mg protein using an extinction coefficient (***ε***) of $1.56\times10^{5}M^{-1}\ cm^{-1}$.

The GSH and GSSG contents in lung perchloric acid supernatant were determined by the DTNB-GSSG reductase recycling assay in triplicate as described before (Pontes et al., 2008) with some modifications. Briefly, the thawed acidic supernatant was neutralized with equal volume of 0.76 M $KHCO_3$ and centrifuged for 10 min at 13,000g (4 ºC). For measurement of GSHt, 100 µL/well of the neutralized supernatants, standards or blank were added in triplicate to 96-well microtiter plates, followed by 65 µL/well of freshly prepared reagent containing 0.69 mM NADPH and 4 mM DTNB in 72 mM phosphate buffer. Plates were then incubated at 30 ºC in a plate reader (BioTek Instruments, Vermont, US), for 15 min prior to the addition of 40 µL/well of a 10 IU/mL glutathione reductase solution in phosphate buffer. The stoichiometric formation of 5-thio-2-nitrobenzoic acid was followed for 3 min at 415 nm and compared to a standard curve. For the determination of GSSG, 10 µL of 2-vinylpyridine were added to 200 µL aliquots of acidic supernatant and mixed continuously for 1 h for derivatization of GSH. GSSG was then measured as described above for total glutathione. The molar GSH levels were calculated by subtracting the GSSG content from the total glutathione content (GSH = GSHt – 2 × GSSG).

Lung MPO activity was measured according to a previously described assay (Andrews and Krinsky, 1982) with slightly alterations. Briefly, the resulting supernatant was solubilized in hexadecyltrimethylammonium bromide (1:1 v/v) to a final concentration of 0.5% (w/v). Then, the samples were sonicated to disrupt the granules and solubilize the MPO and afterwards centrifuged at 4,000g at 4 ºC for 5 min. The assay mixture consisted of 75 µL sample, blank or standard, 75 µL of TMB (1.6 mM), dissolved in dimethyl sulfoxide and 525 µL of assay buffer (220 mM phosphate, pH 5.4, with 110 mM NaCl). The enzymatic activity was initiated by adding H_2O_2, 3 mM, and evaluated at 650 nm at 37◦C during 3 min. MPO standards were used and the results were expressed U/g of protein.

Paraquat quantification

Quantification of PQ in plasma, urine, lung tissue and feces was performed following a previously described method (Moreira et al., 2012). Briefly, an aliquot of 0.5mL of each aqueous supernatant, 1.5 mL of phosphate buffered saline solution (pH 8.0) and 20 µL of the internal standard [ethyl paraquat (EPQ) solution (100µg/mL)] were pipetted into a 15mL glass tubes. Ten milligrams of $NaBH_4$ were added to the mixture in order to reduce PQ and the EPQ to the hydrogenated derivatives (HPQ and HEPQ) accordingly to the method described (Draffan et al., 1977). The reduction was left to occur at 60°C for 10 min and the mixture was then kept in cold water for 2 min. SPE was performed using Bond Elut® C18 cartridges installed on a vacuum manifold. The cartridges were preconditioned with 2mL of methanol and 2mL phosphate buffer (pH 8). The sample was transferred to the SPE column, which was washed with 2mL of deionized water. Afterwards, PQ was eluted under vacuum, at a flow/rate of 1.5 mL/min with 2mL of methanol. The eluate was evaporated at room temperature under a gentle stream of

nitrogen. The residue was reconstituted in 100 µL of methanol and 2µL was injected in the GC-IT/MS system (Moreira et al., 2012). GC-IT/MS analyses were performed using a Varian CP-3800 GC (USA) gas chromatographer equipped with a Varian Saturn 4000 ion trap mass detector (USA) and a Saturn GC/MS workstation software version 6.8. The GC was equipped with a VF-5ms (30m×0.25mm×0.25 µm film thickness) from Varian. The carrier gas was Helium C-60 (from Gasin, Portugal) at a constant flow rate of 1 mL/min. The temperature of the injector port was 250°C and it operated in split mode 1:10. The initial column temperature was maintained at 80°C for 1 min; programmed at 10°C/min to 200°C and 20°C/min to 270°C with a hold at 270°C for 5 min. All mass spectra were acquired by electron impact (EI, 70 eV) in full-scan mode. The ion-trap detector was set as follows: the transfer line, manifold and trap temperatures were 280, 50 and 180°C, respectively. The mass range was 50–600 m/z, with a scan rate of 6 scan/s. The emission current was 50 µA, and the electron multiplier was set in relative mode to autotune procedure. The maximum ionization time was 25000 µs, with an ionization storage level of 35 m/z. Chromatographic peaks (for retention time evaluation) and ions for HPQ qualitative and quantitative analysis were initially selected from the analysis of pure standards (i.e. without matrix). Confirmation of the ions was done by the analysis of spiked blank samples with a known PQ concentration in a full scan chromatogram (FSC) mode. Ions with significant abundance were vectored as either target or qualifier ions for the selected ion monitoring analysis. The designated ions were m/z 96, 148, 192 (HPQ) and 110, 162 and 220 (HEPQ).

Tissue processing for structural analysis

Cubic pieces from the right upper lobe, right middle lobe and right lower lobe of the lung were fixed [4% (vol/vol) buffered paraformaldehyde] by diffusion, during 24 h, and subsequently dehydrated with graded ethanol and included in paraffin blocks. Xylene was used in the transition between dehydration and impregnation. Serial sections (5 µm of thickness) of paraffin blocks were cut by a microtome and mounted on silane-coated slides. The semiquantitative histopathological evaluation of acute lung damage was performed according to a previously described procedure (Dinis-Oliveira et al., 2006a; Dinis-Oliveira et al., 2006b). The following parameters were analyzed for each group: (i) tissue disorganization, (ii) inflammatory reaction, (iii) necrotic zones, and (iv) interstitial fibrosis. The severity of tissue disorganization was scored according to the percentage of the affected tissue: score 0 = normal structure; score 1 = less than one-third of tissue; score 2 = greater than one-third and less than two-thirds; score 3 = greater than two-thirds of tissue. The severity of inflammatory reaction was scored as follows: grade 0 = no cellular infiltration; grade 1 = mild leukocyte infiltration (1 to 3 cells by visual field); grade 2 = moderate infiltration (4 to 6 leukocytes by visual field); and grade 3 = heavy infiltration by neutrophils. The severity of necrosis was scored as follows: grade 0 = no necrosis; grade 1 = dispersed necrotic foci; grade 2 = confluence necrotic areas; grade 3 = massive necrosis. The interstitial fibrosis was scored from 0 (normal lung) to 8 (total

fibrosis) according to the following criteria: grade = 0 normal lung; grade 1 = minimal fibrous thickening of alveolar or bronchial walls; grades 2–3 = moderate thickening of walls without obvious damage of lung architecture; grades 4–5 = increased fibrosis with definite damage to lung architecture and formation of fibrous bands or small fibrous mass; grades 6–7 = severe distortion of structure and large fibrous areas; "honeycomb lung" was placed in this category; grade 8 = total fibrotic obliteration of the field (Dinis-Oliveira et al., 2007b).

Staining procedures

The slides were dewaxed in xylene and hydrated through graded alcohols finishing in phosphate-buffered saline (10mM PBS, pH 7.2). Deparaffinised sections were stained with haematoxylin-eosin, and others were prepared for immunohistochemistry NF-κB analysis, accordingly to the previous described study (Dinis-Oliveira et al., 2009b). Briefly, the haematoxylin-eosin staining was performed by immersion slides in Mayer's haematoxylin solution for 3–4 min followed by immersion in 1% eosin solution for 7 min, dehydration with graded alcohols through xylene, and mounting with DPX. To perform the NF-κB immunohistochemistry detection, after deparaffinization and rehydration of the slides, endogenous peroxidase activity was blocked with 3% H_2O_2 in Tris-buffered saline (TBS) and methanol for 15 min, followed by 2×5 min washes with 0.1% TBS with Tween 20 (TTBS). To reduce nonspecific binding, slides were incubated with blocking solution (3% BSA in TTBS) for 1h30. Next, sections were incubated for 3h with the primary antibody directed to NF-κB (NFκB p50 rabbit polyclonal antibody; 1:50, sc-114; Santa Cruz Biotechnology). Sections were then washed 2×5 min with TTBS and incubated with secondary antibody (goat anti-rabbit IgG-horseradish peroxidase; 1:100, ab6721; abcam) for 2 h at room temperature. 3, 3´-Diaminobenzidina was used as the precipitating substrate for the localization of peroxidase activity (DAB Substrate Kit ab64238; 1min incubation RT).

Statistical analysis

All statistical calculations were performed with the GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California, USA). The Shapiro-Wilk test was performed to check normality of the data. Statistical comparisons among groups were estimated using the one-way ANOVA (in case of normal distribution) followed by the Student Newman Keuls *post hoc* test or Kruskal-Wallis (in case of distribution is not normal). Data from light microscopy analysis were expressed as median and interquartile range and the statistical comparisons were performed using Kruskal-Wallis.

In all cases, *p* values lower than 0.05 were considered as statistically significant. All data obtained from the results of the biochemical assays and PQ quantifications were expressed as mean±SD (standard deviation)

Results

Determination of the acute oral toxicity by the up-and-down (UDP) procedure

The progression of the toxicological signs after dosing was notoriously different between PQ and PQ+LAS groups. The dose progression is shown in Table 1 and 2. The estimated LD50 values were calculated by the

AOT software and were 34.86 mg/kg (95% CI 40–125 mg/kg) and 187.5 mg/kg (95% CI 125–400 mg/kg), respectively. The animals that received 125 mg/kg of PQ+LAS did not show any signs of toxicity and fully survived more than 14 days. On the other hand, animals intoxicated with PQ 125 mg/kg were prostrated after dosing and 24h later they showed other signs of toxicity such as epistaxis, piloerection, cyanosis and respiratory distress. All the animals from this group died in a period between 3 to 14 days post dosing probably due to respiratory failure.

Table 1. Dose progression given with short-term (48 h) and long-term (14 days) fates of individual female Wistar rats dosed orally with Gramoxone®

Test animal	Gender	Dose mg/kg	Short term (48h)	Long term (14 days)
1	female	40	O	O
2	female	125	O	X
3	female	400	X	X
4	female	125	O	X
5	female	400	X	X
6	female	125	O	X
7	female	400	X	X

X = Died, O = Survived

Table 2. Dose progression given with short-term (48 h) and long-term (14 days) fates of individual female Wistar rats dosed orally with the new formulation PQ+LAS

Test animal	Gender	Dose mg/kg	Short term (48h)	Long term (14 days)
1	female	40	O	O
2	female	125	O	X
3	female	400	X	X
4	female	125	O	X
5	female	400	X	X
6	female	125	O	X
7	female	400	X	X

X = Died, O = Survived

Acute toxicity at the end points 24h and 48h

Rats exposed to PQ formulation showed histological and biochemical changes in blood and lungs. The protection of LAS was more pronounced at the end point 48h. Noteworthy, for the PQ 48h were necessary 7 animals, since 2 rats from this group died before the 48h. Rats intoxicated with the new formulation survived until the end point of the protocol.

Biochemical analysis in plasma and urine

As shown in Fig. 1, animals from PQ 24h group exhibited a significant increase of NAG levels in urine compared to control group (18.42±2.42 vs 9.23±5.08, $p < 0.05$). On the other hand, this parameter was kept near to control levels in the PQ+LAS group (10.80±3.07). Analogous results were obtained for protein content, while for the clearance of creatinine it was observed the inverse effects, in accordance with the NAG levels (Fig. 1). Plasma biochemistry quantified 24h after intoxication only showed slight variations (Table S1). On the other hand, several plasma biochemical parameters evaluated 48h after administration varied significantly, as shown in Table 3. Conjugated bilirubin, glucose, cholesterol, LDL, GGT, and CK levels were significantly increased in the PQ group, while this effect was reverted in the PQ+LAS group. On the other hand, creatinine, ALP, CK-MB, α-1-antitrypsin and calcium levels were increased in PQ and PQ+LAS groups. Conversely, the animals exposed to PQ, PQ+LAS and LAS had lower levels of HDL compared to the control group.

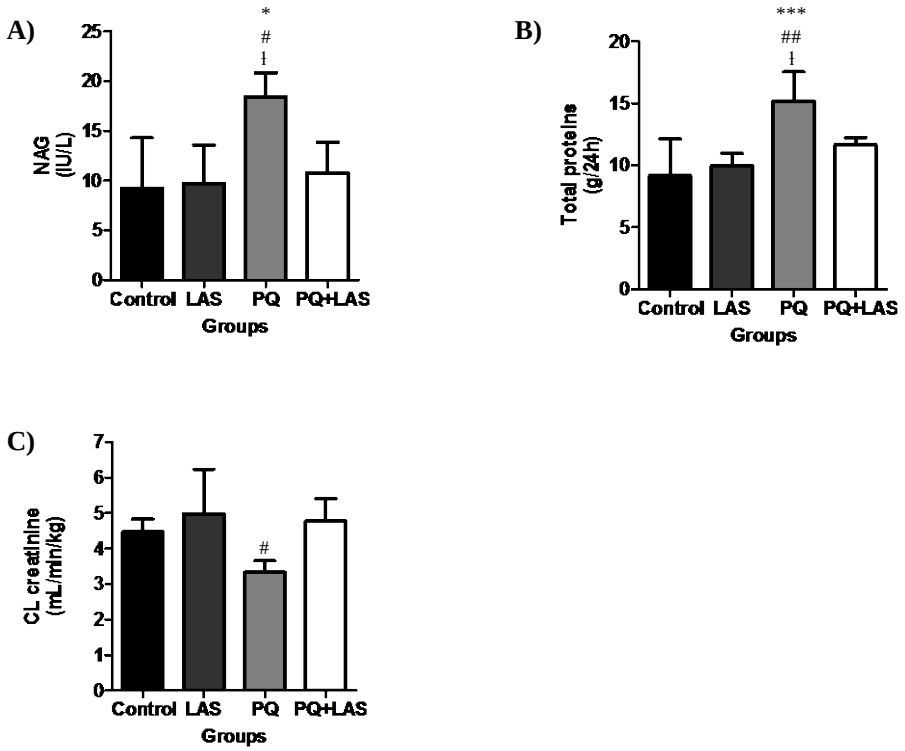

Figure 1. Biomarkers of kidney injury after 24h. A) Urinary *N*-acetyl-β-D-glucosaminidase (NAG) activity in the control, lysine acetylsalicylate (LAS), paraquat (PQ), and paraquat plus lysine acetylsalicylate (PQ + LAS) groups. B) Total proteins in the control, LAS, PQ, and PQ+LAS groups. C) Clearance of creatinine in the control, LAS, PQ, and PQ+LAS groups. Values are given as mean±S.D. (n = 5). $^{*}p< 0.05$, $^{**}p< 0.01$ and $^{***}p< 0.001$ vs. control; $^{\#}p< 0.05$, $^{\#\#}p< 0.01$ vs. LAS; $^{ł}p< 0.05$ vs. PQ+LAS.

Pulmonary oxidative stress and inflammatory biomarkers

In view of the fact that PQ produces ROS during the redox cycling, several pulmonary oxidative stress and inflammatory biomarkers were also evaluated. The lung GSH and TBARS levels did not change 24h after intoxication among groups (data not shown). However, 48h after intoxication there was a significant depletion of lung GSH levels in the PQ groups that correspond to a depletion of approximately 40% compared to control (Fig.2A). In accordance, a significant increase of approximately 85% was observed in the GSSG levels of the PQ-treated group (Fig. 2B). In accordance, the GSH/GSSG ratio suffered a significant reduction in the PQ group compared to the control group (Fig. 2C). In both cases, LAS attenuated the induction of oxidative stress shown by the normalization of GSH and GSSG levels. Likewise, pulmonary lipid peroxidation was exacerbated in PQ group (Fig 2D) and a significant decrease in TBARS levels to normal was observed in the lung of animals from PQ+LAS group. Concerning MPO, it was observed that this inflammatory parameter increased at 24h in the PQ group, but the presence of LAS did not modify this trend. On the other hand, at 48h, the differences among groups were no longer apparent (Fig. 3).

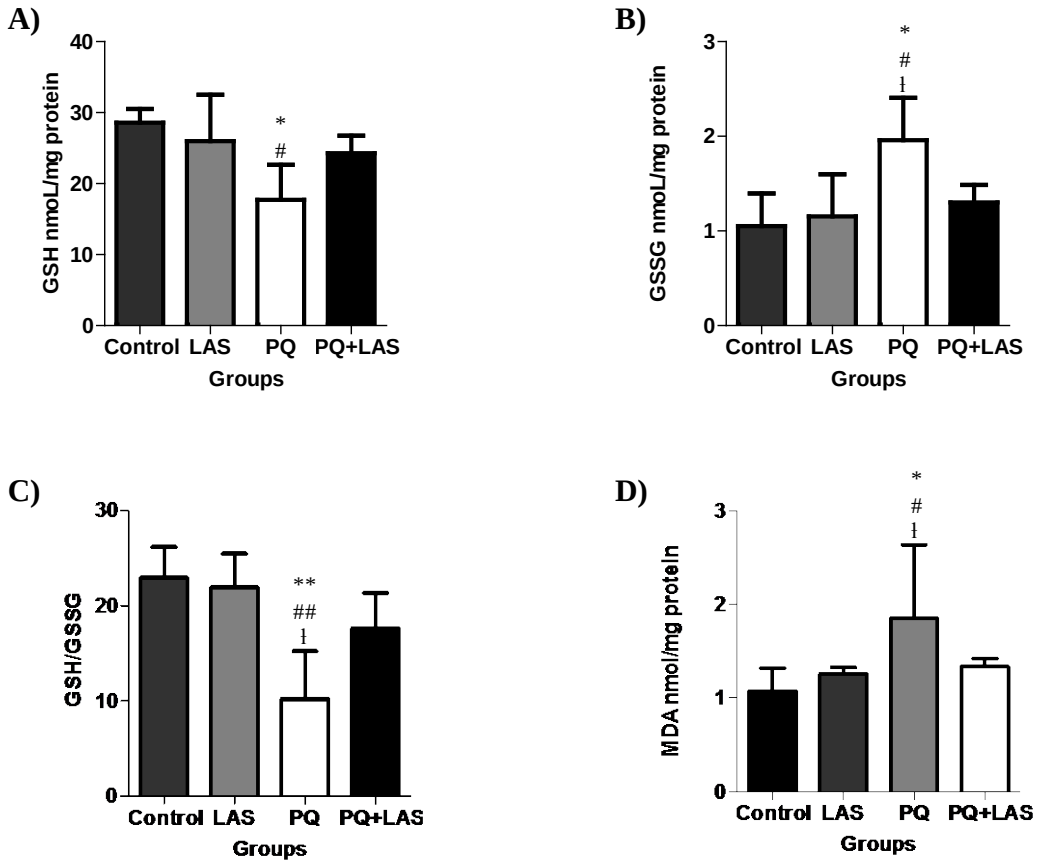

Figure 2. GSH (nmol GSH/mg of protein), GSSG (nmol GSSG/mg of protein), GSH/GSSG ratio and malondialdehyde (MDA nmol/mf of protein) in the control, PQ, LAS, PQ, and PQ+LAS groups. Values are given as means±S.D (n=5). $^{*}p< 0.05$, $^{**}p< 0.01$ vs. control; $^{\#}p< 0.05$, $^{\#\#}p< 0.01$ vs. LAS; $^{ł}p< 0.05$ vs. PQ+LAS.

Table 3. Plasma biochemistry following 48h of intoxication.

Evaluated parameter	Control	LAS	PQ	PQ+LAS
Albumin (g/L)	45.91 ± 4.52	36.01 ± 10.44	42.23 ± 4.88	57.30 ± 31.23
Total proteins (g/L)	74.21 ± 3.55	54.56 ± 2.85	59.25 ± 6.33	57.20 ± 1.66
Total Bilirubin (mg/dL)	0.08 ± 0.01	0.12 ± 0.04^{ł}	0.13 ± 0.07^{ł}	0.04 ± 0.02
Direct Bilirubin (mg/dL)	0.18 ± 0.03	0.09 ± 0.07	0.046 ± 0.03	0.02 ± 0.03**
Glucose (mg/dL)	100.32 ± 24.73	114.16 ± 17.58	360.64 ± 51. 98$^{ł ł ł ***\#\#\#}$	223.70 ± 34.74$^{\#\#\#***}$
Creatinine (mg/dL)	0.36 ± 0.06	0.68 ± 0.63	0.60 ± 00*	0.72 ± 0.39*
Urea (mg/dL)	27.15 ± 5.93	28.80 ± 15.23	50.96 ± 19.52	29.64 ± 7.49
Amylase (U/L)	358.80 ± 28.36	314.28 ± 41.78	405.00 ± 91.12	426.32 ± 49.98$^{\#}$
LDH (U/L)	332.30 ± 86.21	485.76 ± 349.68	666.68 ± 263.92	731.32 ± 263.92
Cholesterol (mg/dL)	49.52 ± 9.87	53.56 ± 9.93	82.24±16.53$^{**\#\#ł}$	64.16 ± 6.10
LDL (md/dL)	25.06 ± 6.69	38.78 ± 9.91	60.38 ±19.45$^{**\#}$	45.88 ± 5.70*
HDL (mg/dL)	17.24 ± 3.77	8.32 ± 4.16**	13.20 ± 1.54$^{*\#}$	11.36 ± 1.28$^{*\#}$
Uric acid (mg/dL)	0.52 ± 0.18	0.76 ± 0.43	0.92 ± 0.52	0.80 ± 0.37
TG (mg/dL)	36.12 ± 4.85	32.28 ± 22.86	43.28 ± 13.86	34.60 ± 8.56
ALAT (U/L)	26.40 ± 7.47	26.05 ± 13.95	16.65 ± 8.82	14.28 ± 5.31
ASAT (U/L)	77.08±9.04	102.60±26.41	112.40±69.95	154.30±49.99
GGT (U/L)	0.28 ± 0.15	0.64 ± 0.92	1.72 ± 3.57$^{*\#ł}$	0.16 ± 0.15
ALP (U/L)	60.40 ± 5.90	95.6 ± 38.26	131.20 ± 19.10**	113.60 ± 30.63*
Lactate (mg/dL)	22.79 ± 6.62	22.84 ± 7.74	33.04 ± 6.99	24.15 ± 6.80
α-1-Anti-tripsyn (g/L)	0.07 ± 0.00	0.07 ± 0.04	0.07 ± 0.03	0.09 ± 0.01*
CRP (mg/L)	0.004 ± 0.009	0.042 ± 0.034	0.084 ± 0.188	0.027 ± 0.038
CK (U/L)	562.54±111.34	440.95 ± 259.82	956.10±307.49$^{*\#ł}$	458.50 ± 64.61
CK-MB (U/L)	308.40±76.90	444.55 ± 142.16	677.91 ± 60. 95$^{**\#}$	552.76 ± 109.71*
Potassium (mmol/mL)	4.17 ± 0.33	6.61 ± 1.04**	4.50 ± 0.66$^{\#\#\#}$	4.99 ± 0.29$^{\#\#}$
Calcium (mg/dL)	9.85 ± 2.00	14.00 ± 0.66	14.71 ± 0.85*	15.03 ± 1.53*
Sodium (mmol/mL)	129.76 ± 12.27	180.20 ± 11.56***	165.76 ± 11.19	173.08 ± 8.61

Values are given as mean±S.D. (n = 5); * p < 0.05 *vs*. Control; # p < 0.05 *vs*. LAS; ł p < 0.05 *vs*. PQ+LAS

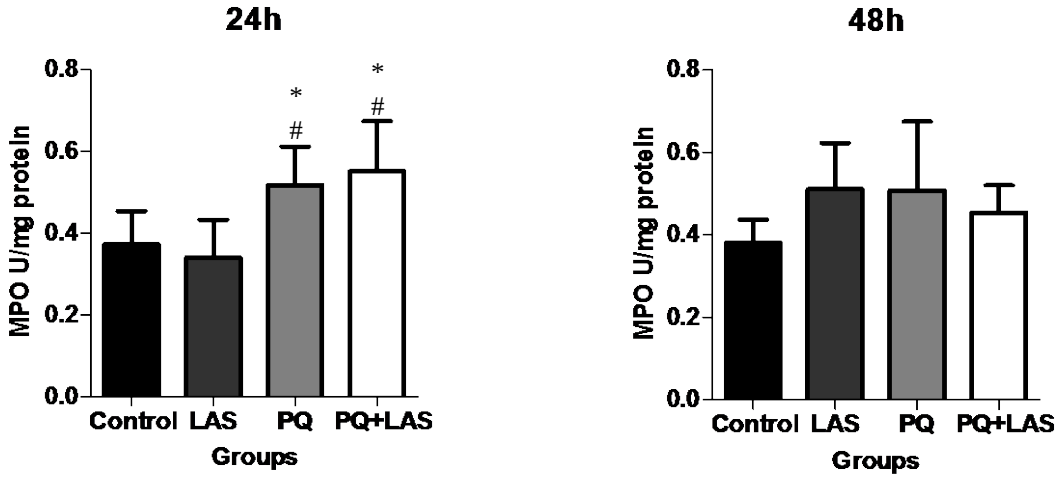

Figure 3. Lung mieloperoxidase (MPO activity (U/mg of protein) of the control, LAS, PQ, PQ+LAS groups. Values are given as means±S.D (n=5). *p < 0.05 vs. control; $^{\#}p$< 0.05 vs. LAS.

Paraquat quantification

The plasma concentration of PQ in the PQ 24h group was 8.14±4.90 µg/mL. Animals treated with PQ+LAS formulation evidenced a significant decrease in plasma PQ concentration, down to 2.80±0.29 µg/mL ($p<0.05$; Fig. 4). Although not significant, a trend for an increase of urinary PQ elimination was registered in the PQ+LAS group. On the other hand, PQ fecal excretion was significantly increased in rats exposed to PQ+LAS compared to the group only exposed to PQ, from 122.45±13.79 µg/mL to 605.25±414.76 µg/mL ($p<0.01$; Fig. 4). No PQ was detected in lung tissue in both groups after 24h. After 48h, the concentration of PQ in lungs of the PQ+LAS-treated group was lower than the PQ-treated group, 0.41±0.07 µg/mL and 1.83±1.03 µg/mL ($p<0.05$), respectively (Fig. 5). In addition, rats treated with PQ+LAS and sacrificed 48h later also had significantly lower levels of PQ in plasma than PQ-treated group (for PQ 1.83±1.03 vs. 0.41±0.06 for PQ+LAS, $p<0.05$). Urine and feces concentration of PQ was unaltered after 48h.

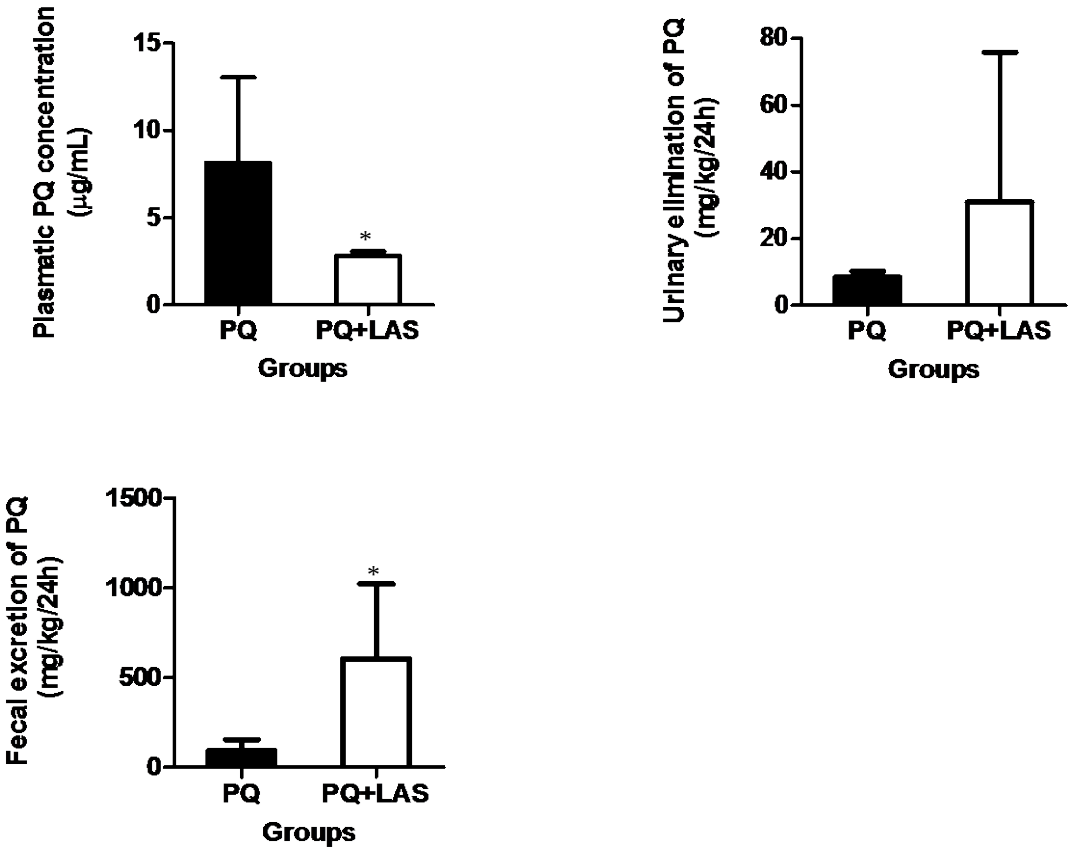

Figure 4. Paraquat (PQ) concentration in the plasma, urine, and feces of PQ 24h and paraquat+lysine acetylsalicylate (PQ+LAS 24h) groups. Values are given as means±S.D (n=5). $^{*}p<0.05$ vs. PQ.

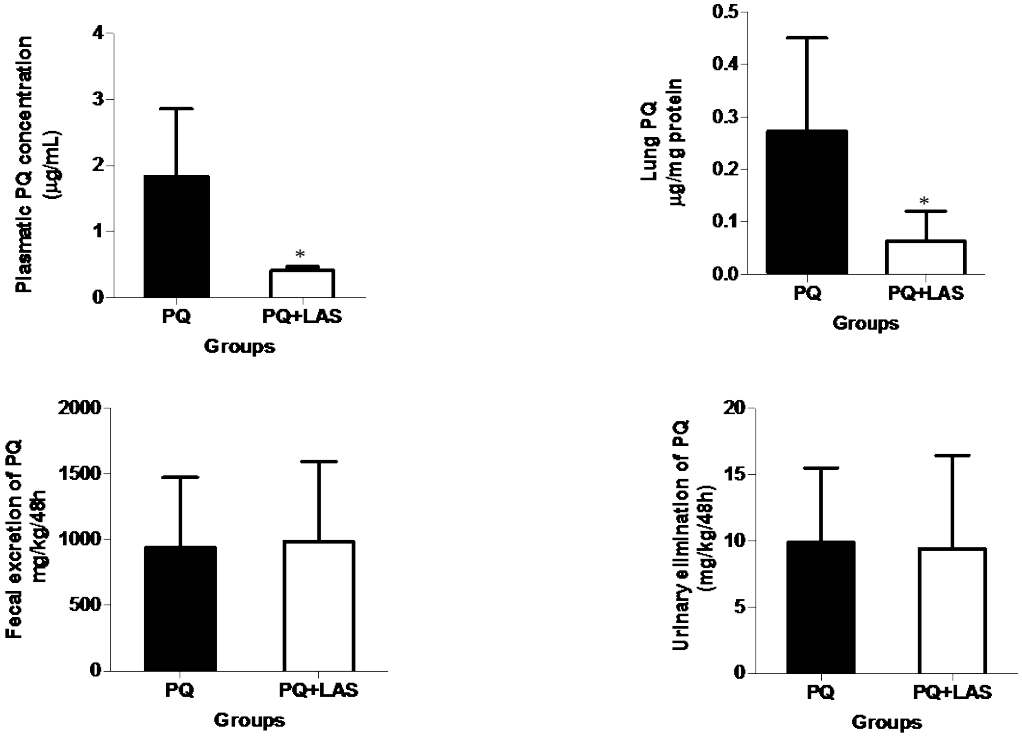

Figure 5. Paraquat (PQ) concentration in the plasma, lung, feces, and urine of PQ 48h and paraquat+lysine acetylsalicylate (PQ+LAS 48h) groups. Values are given as means±S.D (n=5). $^{*}p<0.05$ vs. PQ

Histology

The major histological findings and respective semi-quantitative analysis is shown in Table 4. Animals from the control and LAS group presented a normal and preserved pulmonary architecture, without evidences of alveolar collapse or cellular infiltrations at 24 and 48h (Fig.6 and 7). Lungs from PQ only exposed rats showed marked fibrin deposition in the alveolar space, diffuse alveolar collapse, strong vascular congestion and infiltrative cells within the alveolar space (Fig.6). These histopathological alterations became more exuberant at 48h after PQ exposure, with an extension of interstitial areas occupied by fibrin and trapped erythrocytes, dispersed necrotic foci and inflammatory cells (Fig. 7). Noteworthy, in the PQ+LAS 24 and 48h groups, compared to the PQ-only exposed animals, the incidence of the above referred alterations was drastically attenuated, particularly the macrophage-like cells infiltration, fibrin deposition and the alveolar collapse.

The immunohistochemistry for the evaluation of the lung activation of the inflammatory transcriptional factor, NF-κB in response to PQ and PQ+LAS insult is depicted in Figure 8. Both lungs from PQ 24 and 48h group showed a strong positive staining for activated NF-κB in the macrophage-like cells. On the other hand, in the PQ+LAS 24 and 48h groups the activation of NF-κB was dramatically attenuated. Noteworthy, activated macrophages-like cells were not found.

Table 4. Semi-quantitative histological analysis of the control, lysine acetylsalicylate (LAS), paraquat (PQ) and paraquat plus lysine acetylsalicylate (PQ+LAS) groups.

Group		Evaluated morphological parameter		
		Interstitial inflammatory cell infiltration	Tissue Desorganization	Fibrine deposition
24h	Control	0.0[#]	0.0[#]	0.0[ł]
	LAS	0.0[#]	0.0[#]	0.0[ł]
	PQ	2.0 (2-3)	1.0 (1-2)	1.0 (1-2)
	PQ+LAS	1.0 (0.5-1)[ł]	0.5 (0.25-0.75)	0.5 (0.25-0.75)
48h	Control	0.0[#]	0.0[#]	0.0[#]
	LAS	0.0[#]	0.0[#]	0.0[#]
	PQ	2.0 (2.0-2.5)	1.0 (1.0-1.5)	1.0 (1.0-1.5)
	PQ+LAS	0.25 (0.0-0.625)[ł]	0.0[#]	0.0 (0.0-0.5)[ł]

Values are given as median and interquartile range (n = 3). ł $p < 0.05$ vs. PQ group; [#] $p < 0.01$ vs. PQ group.

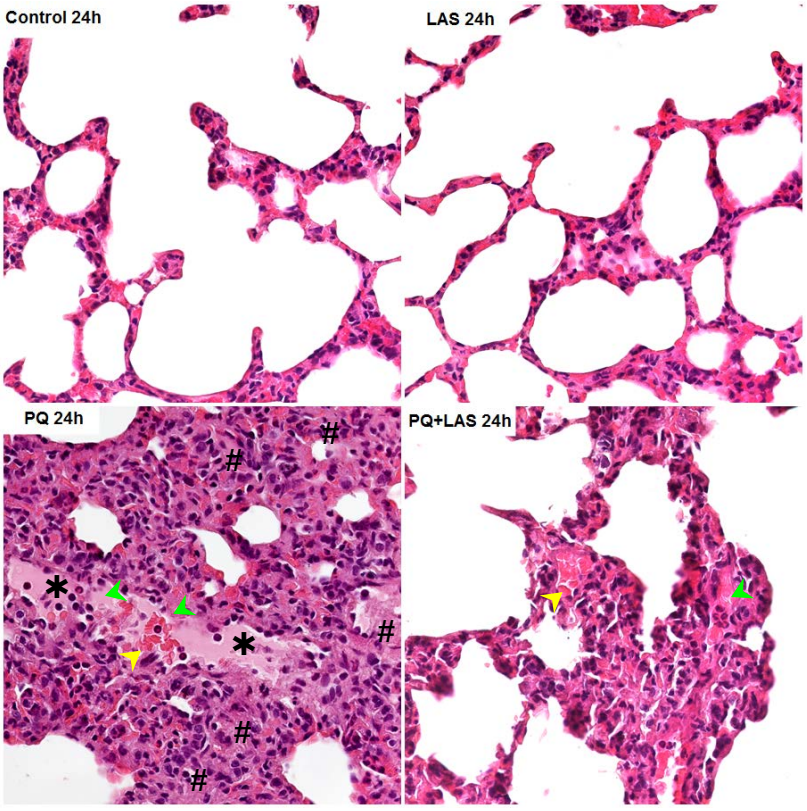

Figure 6. Representative light micrographs of lungs from animals of control 24h, LAS 24h, PQ 24h and PQ+LAS 24h groups. The infiltrative cells (green arrows) along with extensive fibrin deposition (*) and trapped erythrocytes (yellow arrows) within the alveolar space can be observed. It can also be noted few necrotic areas (#) shown by the presence of cellular debris.

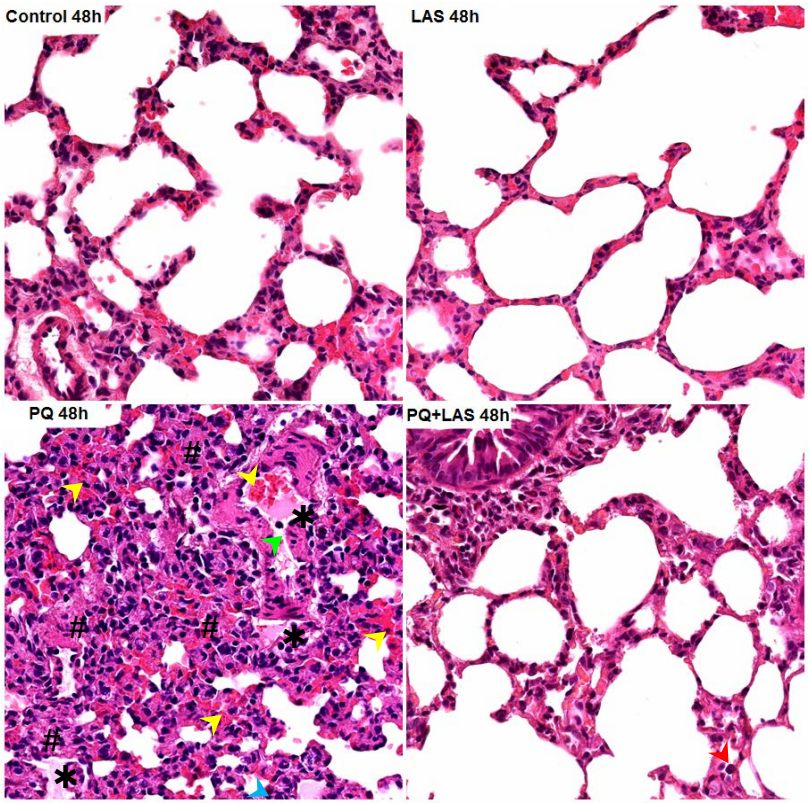

Figure 7. Representative light micrographs of lungs from animals of control 48h, LAS 48h, PQ 48h and PQ+LAS 48h groups. The infiltrative macrophages (blue arrows) adherent to endothelium and other inflammatory cells (green arrows) within the vases can be observed. It can also be observed capillaries filled with erythrocytes, suggestive of vascular stasis (yellow arrows) and extended interstitial areas filled with fibrin (*) and dispersed necrotic foci (#).

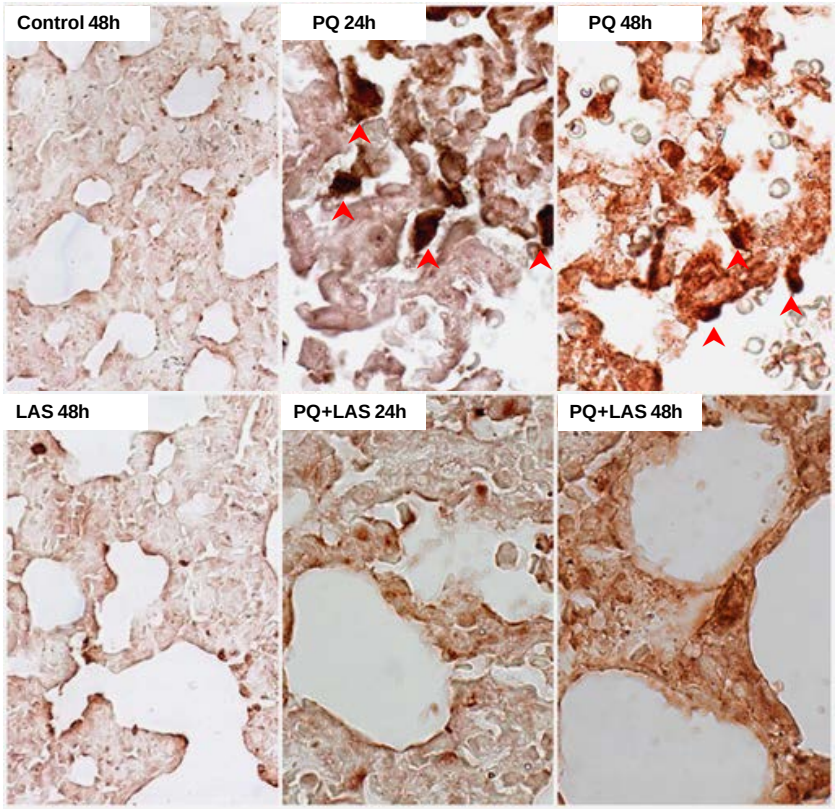

Figure 8. Light micrographs of lung NF-κB immunohistochemistry from animals belonging to the control, LAS, PQ and PQ+LAS groups, sacrificed 24 and 48 h after the administrations. NF-κB activation is evident in macrophage-like cells (red arrows).

Discussion

The present study provides unequivocal evidence that the addition of LAS to the PQ commercial formulation (Gramoxone®) ameliorates the signs of PQ induced-toxicity in Wistar rats. The current findings corroborate previous results that demonstrated the improvement of the safety profile of this new formulation, as shown by the full survival of male Wistar rats (Baltazar et al., 2013a). Accordingly, it was now shown that the PQ+LAS formulation has a much higher LD50 than the LD50 of Gramoxone® (34.86 mg/kg *vs*. 187.5 mg/kg).

In the past few decades, a considerable amount of research was performed in order to discover new therapeutic solutions for the burden associated with PQ poisonings (Dinis-Oliveira et al., 2008b). Measures to alter the toxicokinetics of PQ were the first to appear. PQ is known to be very rapidly absorbed, apparently associated with the carrier-mediated transport system for choline on the brush-border membrane (Nagao et al., 1993). PQ distribution follows a three-compartment model where the third compartment is the lung, especially the pneumocytes type I and II and Clara cells, in which exchanges with the central compartment (blood) are slow (Hawksworth et al., 1981). Peak concentrations of PQ in the lung are reached 5-7 h after ingestion and lethal concentrations may be achieved within 6h of ingestion of 35 mg/kg (Houze et al., 1990). Since patients are

rarely hospitalized within this time, the extracorporeal elimination techniques will be effective in lowering PQ plasma levels but with low effectiveness in improving the survival rate. The reason is that when these techniques of elimination are initiated, potentially lethal concentrations of PQ have already been reached in the highly vascular tissues of vital organs and in pneumocytes (Bismuth et al., 1987). Administration of other drugs aiming to control the damage caused by PQ, such as dexamethasone, cyclophosphamide and N-acetylcysteine also tend to fail to improve the survival rate for the same reason, since most of the damage is already irreversible by the time of the treatment (Eddleston et al., 2003). Heylings and colleagues initiated the first attempts to make the product safer, whether the poisoning occurs accidentally or intentionally. The new formulation developed by this group contained an alginate that has the unique property that it gels on contact with gastric acid, decreasing the dispersion and delivery of the toxic chemical to its site of absorption in the small intestine (Heylings et al., 2007). However, as already mentioned, this formulation had a negligible impact in the survival rate. Our attempt to make Gramoxone® safer was to include LAS in the original formulation that, immediately after ingestion, would protect the gastrointestinal tract and counteract the toxicity of the absorbed PQ (Baltazar et al., 2013a). This measure, that does not influence the herbicidal effect (Baltazar et al., 2013a), has the potential to overcome the critical period between poisoning and hospital treatment by preventing lethal concentrations to reach the lung and even supposing that lethal concentrations accumulate in this organ, LAS would immediately exert its cytoprotection.

The 24h results have shown that LAS alters the toxicokinetics of PQ by increasing the PQ elimination in urine and feces, decreasing plasma concentration. The results suggest that the alteration might be related to the improvement of the renal function, as sustained by the restoration of the clearance of creatinine and the levels of the lysosomal enzyme NAG, a tubular nephrotoxicity marker. Besides lung, kidney injuries are major causes of PQ morbidity, since it is mainly excreted unchanged by the kidney (Kim et al., 2011; Kim et al., 2009; Roberts et al., 2011). Renal dysfunction leads, in turn, to decreased renal PQ clearance, which promotes greater toxicity in other organs. After 48h of intoxication, the results were similar for the PQ plasmatic concentrations, and PQ was already detectable in the lung but at a lower extent in the PQ+LAS group. In acute PQ poisoning, creatinine peaks around five days post-ingestion and resolves within three weeks in survivors (Kim et al., 2009). This fact may explain in part the reason why in the present study a rise of creatinine is not observed in the PQ group at the end of 24 and 48h. A recent preclinical model explored a variety of urinary and plasma biomarkers and their possible roles in the prediction of acute kidney injury following administration of sub-lethal PQ doses (Wunnapuk et al., 2013). The authors found that urinary kidney injury molecule-1, urinary albumin and urinary Cystatin-C elevations outperformed plasma creatinine in predicting histological changes at 8–24 h. Other explanation for the reduced levels of lung PQ in the group PQ+LAS 48h might arise from a direct interference with the

mechanism of PQ uptake by the cells. The substrates of the polyamine uptake system require specific structural characteristics such as: (i) two or more positively charged nitrogen atoms, (ii) maximum positivity of charge surrounding these nitrogens, (iii) a nonpolar group between these charges, and (iv) a minimum of steric hindrance (Ross and Krieger, 1981). Evidences suggest that LAS metabolite, salicylic acid (SA), forms crystalline charge-transfer complexes with PQ dichloride (Dinis-Oliveira et al., 2008a). Therefore, it could be hypothesized that the portion of PQ complexed with LAS would not be recognized by the polyamine uptake system, thus limiting its internalization.

The pharmacokinetic studies have shown that apart from the lung, the muscle is the other main reservoir of PQ (Smith, 1988) and for that reason muscle toxicity is expected. *Postmortem* analysis have shown that heart muscle and the diaphragm presented cellular degeneration, including changes of cross striation, vascular congestion, interstitial edema, localized hemorrhagic infiltration and numerous marginalized leukocytes within capillaries (Dinis-Oliveira et al., 2009b). Several authors reported elevated plasma CK levels (1147 -1796 U/L), increased PQ muscle concentration, and damage (Rose et al., 1976; Sharp et al., 1972) four or five days after admission (Tabata et al., 1999; Van de Vyver et al., 1985). Accordingly, our data revealed that the plasma CK values were elevated after 24h and 48h of administration of PQ (1383 and 956 U/L, respectively). On the other hand, LAS administration exerted a beneficial effect since the levels of CK were significantly lower after 48h (956.10±307.49 vs. 458.50±64.61 U/L, respectively).

PQ acute fulminant toxicity is characterized by an elevation of the pancreatic enzymes (Bismuth et al., 1982) and mild acute pancreatitis was observed at autopsy of PQ-poisoned patients (Soontornniyomkij and Bunyaratvej, 1992). Accordingly, in our experimental model it was observed an increase of the pancreatic enzyme, amylase in both groups PQ 24h and PQ+LAS 24h, while at 48h the increase was only statistical significant for the group PQ+LAS 48h. Early animal experiments have shown that PQ increases blood glucose and decreases plasma insulin (Giri et al., 1979). The mechanism underlying PQ induced-hyperglycemia might be related to the inhibition of the activities of phosphatidylinositol 3´-OH kinase (PI 3-kinase) bound to insulin receptor substrates and phosphorylation of Akt (Shibata et al., 2010). This kinase has been shown to play an essential role in insulin dependent translocation of GLUT4 to the plasma membrane, leading to the impairment of glucose uptake by adipocytes (Kimura et al., 2010; Shibata et al., 2010). The hyperglycemic effect observed in PQ-treated rats was attenuated by LAS only in the PQ+LAS 48h, probably due to a direct effect of LAS in lowering glucose levels rather that an amelioration of the pancreas toxicity since the amylase levels were unchanged. In fact, case reports suggested that high-dose NaSAL could diminish glycosuria in diabetic patients and plasmatic glucose concentrations (Reid et al., 1957; Williamson, 1901). The transcription factor nuclear factor kappa B (NF-*κ*B) is activated in response to several oxidant and inflammatory stimuli and promotes the activation of downstream effectors not only

involved in the inflammatory response but also promoting insulin resistance (Shoelson et al., 2003; Tanti and Jager, 2009). The salicylates mediated-reversal of hyperglycemia, hyperinsulinemia, and dyslipidemia is associated with the ability of salicylates to inhibit IKK-β and NF-κB (Kopp and Ghosh, 1994; Yin et al., 1998) and therefore ameliorate insulin resistance and improve glucose tolerance (Cai et al., 2005; Hundal et al., 2002; Yuan et al., 2001).

The main target organ for PQ toxicity is the lung as a consequence of its accumulation, against a concentration gradient, through the highly developed polyamine uptake system. Once inside the cells, PQ^{2+} is reduced to the PQ monocation free radical ($PQ^{\bullet+}$), which is rapidly reoxidised by O_2 to PQ^{2+}, initiating a series of reactions leading to production of superoxide anion ($O_2^{\bullet-}$). As expected, the oxidative stress biomarkers were markedly increased in PQ-treated groups namely GSSG and lipid peroxidation. The protection conferred by salicylates against PQ toxicity has been postulated to be, at least in part, due to their antioxidant properties (Baltazar et al., 2011). PQ leads to the generation of $O_2^{\cdot-}$ that when dismutated originates hydrogen peroxide (H_2O_2) which in turn, in the presence of Fe (II) is reduced to $HO^{\cdot}$, a far more damaging ROS (Dinis-Oliveira et al., 2008b). Acetylsalicylic acid and mainly its SA metabolite strongly scavenge $HO^{\cdot}$ and chelate transition metals such as Fe (III) and Cu (II), thus inhibiting the Fenton reaction (Grootveld and Halliwell, 1986). Additionally, acetylsalicylic acid has unique biological functions namely the induction of ferritin and of 15-epi-lipoxin A4 synthesis, NO up-regulation, hemoxygenase-1 expression and inhibition of NAD(P)H oxidase activity (Baltazar et al., 2011).

As mentioned above, an important target is the nuclear factor NF-κB, a pivotal transcription factor involved in the regulation of the expression of proinflammatory genes encoding various cytokines, chemokines, enzymes (e.g., iNOS, COX-2), and adhesion molecules. PQ-induced oxidative stress strongly activates NF-κB in the lung cells of rats intoxicated with PQ (Dinis-Oliveira et al., 2007b). As expected, lung NF- κB was strongly activated in both PQ groups and the incorporation of LAS to the formulation markedly suppressed the PQ-induced lung NF-κB activation. These results are in accordance with previous studies reporting that SA and acetylsalicylic acid inhibit the activation of NF-κB after inflammatory insults such as LPS (Kopp and Ghosh, 1994), or after PQ intoxication (Dinis-Oliveira et al., 2007b). During the inflammatory process, platelets undergo chemotaxis, release proinflammatory cytokines and adhesive proteins, and recruit of neutrophils and macrophages (Manning et al., 1995; Wagner and Burger, 2003). The histological findings also show that LAS inhibited the chemotaxis of inflammatory cells that might be explained either by the inhibition of the adhesion molecule P-selectin or due to the antiplatelet beneficial effects of LAS (O'Sullivan and Michelson, 2006).

The present study shows that the PQ+LAS formulation has the potential to reduce the risks associated with PQ intoxication in mammals, as demonstrated by the amelioration of the overall pathophysiology of PQ poisoning. It is important to focus that the improvement in the survival rate previously

reported (Baltazar et al., 2013a), together with the current findings, support the use LAS in the PQ formulation, as the PQ herbicidal effect is maintained (Baltazar et al., 2013a) and the environmental impact is also attenuated (Baltazar et al., 2013b). Noteworthy, these results were obtained upon the administration of one single dose without other supportive measures. It might be supposed that repetitive LAS therapy could result in survival or at least to extend the survival time enough to allow lung transplantation in the PQ-poisoned patients in worst case approaches.

Acknowledgments

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Supplementary Table

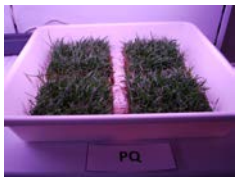
Table S1. Plasmatic biochemical parameters after 24h of intoxication

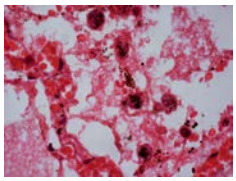
Evaluated parameter	Control	LAS	PQ	PQ+LAS
Albumin (g/L)	42.65 ± 1.37	39.55 ± 13.13	43.23 ± 4.71	46.15 ± 3.74
Total proteins (g/L)	56.50 ± 4.42	64.98 ± 9.45	59.55 ± 6.96	66.43 ± 6.01
Total Bilirubin (mg/dL)	0.060 ± 0.000	0.040 ± 0.028	0.044 ± 0.017	0.027 ± 0.012
Direct Bilirubin (mg/dL)	0.068 ± 0.011	0.064 ± 0.017	0.072 ± 0.027	0.047 ± 0.012
Glucose (mg/dL)	144.20 ± 31.43	121.70 ± 42.71	247.80 ± 80.50*#	257.20 ± 73.91*#
Creatinine (mg/dL)	0.48 ± 0.11	0.52 ± 0.11	0.60 ± 00ł	0.40 ± 0.00
Urea (mg/dL)	34.92 ± 4.24	32.32 ± 10.34	31.20 ± 5.34	35.35 ± 7.52
Amylase (U/L)	367.70 ± 38.86	358.40 ± 45.27	425.90 ± 78.80ł	565.30 ± 243.20*#
LDH (U/L)	468.90 ± 303.10	512.90 ± 277.90	1065.00 ± 903.40	1512.00 ± 443.40
Cholesterol (mg/dL)	44.40 ± 8.57	45.08 ± 3.89	63.12±15.66**#	46.84 ± 9.37
LDL (md/dL)	27.28 ± 5.54	30.85 ± 3.53	48.99 ±14.57*ł#	27.39 ± 9.65
HDL (mg/dL)	9.05 ± 1.69	8.66 ± 1.98	13.78 ± 2.67*#	10.48 ± 2.86
Uric acid (mg/dL)	1.28 ± 0.50	2.36 ± 0.97	2.64 ± 1.04	3.80 ± 1.69*
Triglycerides (mg/dL)	23.88 ± 4.90	27.84 ± 6.83	20.88 ± 12.65	31.70 ± 17.87
ALAT (U/L)	22.24 ± 4.06	29.36 ± 9.38	30.48 ± 4.86	34.20 ± 14.01
ASAT (U/L)	77.08 ± 10.10	102.60 ± 29.52	112.40 ± 78.20	154.30 ± 57.72
GGT (U/L)	0.10 ± 0.00	n.d.	0.70 ± 0.00	0.30 ± 0.00
ALP (U/L)	12.00 ± 6.16	10.80 ± 1.92	7.60 ± 2.88	9.75 ± 2.06
Lactate (mg/dL)	22.79 ± 6.62	26.13 ± 6.63	33.98 ± 11.66	37.64 ± 4.43*
α-1-Anti-tripsyn (g/L)	0.032 ± 0.011	0.060 ± 0.020	0.044 ± 0.022	0.064 ± 0.026
Reactive C protein (mg/L)	0.072 ± 0.058	0.024 ± 0.022	0.068 ± 0.059	0.025 ± 0.030
CK (U/L)	381.70±184.60	673.40 ± 336.00	1383.00 ±1182.00	1340.00 ± 810.70
CK-MB (U/L)	361.10 ± 135.40	380.40 ± 158.00	1071.00 ± 894. 00	1058.00 ± 604.60
Sodium (mmol/mL)	109.90 ± 4.43	100.40 ± 44.17	101.80 ± 8.53	100.80 ± 7.98

Values are given as mean±S.D. (n = 5). n.d: not detectable. * $p < 0.05$ *vs*. control; # $p < 0.05$ *vs*. LAS; ł $p < 0.05$ *vs*. PQ+LAS.

CHAPTER V

INTEGRATED DISCUSSION

PQ is among the most widely used herbicides for weed control management. Its adverse health effects are well recognized, and industrialized countries such as USA, Canada and Australia that allow PQ use, classify it as of "restricted use," which means that it can be used only by people who are licensed applicators. Personal Protective Equipment requirements for those applying paraquat include a long-sleeved shirt and long pants, chemical-resistant gloves, shoes and socks. On the other hand, in developing countries these conditions cannot be guaranteed and despite occupational exposure is not believed to pose a considerable risk, unintentional fatalities still occur (Garnier et al., 1994; Wesseling et al., 1993; Wesseling et al., 1997; Zhou et al., 2013). When it was first marketed, the reddish-brown color led to PQ products being mistaken for beverages such as coffee and coke (prior to 1988), particularly when decanted to other containers – a common practice in developing countries. However, since 1988 the manufacturer Syngenta has changed the color to blue, added an emetic, and a stenching agent to prevent the accidental and intentional ingestions from occurring. U.S. Poison Control Center data, showed a decline of almost 50% when comparing the proportion of all pesticide intoxication cases due to paraquat ingestion for the four years pre- and post-1988 (Wesseling et al., 2001). Although not all the brands marketed have these safeguards added, in USA only formulations with these agents are permitted.

Following the global controversial regarding PQ use, the ban in Europe (explained in commentary I, from the theoretical background chapter of this dissertation) campaigns towards its complete phase-out in developing countries by the Berne Declaration, Pesticide Action Network Asia Pacific, Pesticide Action Network UK and the Swedish Society for Nature Conservation, Syngenta has made some efforts to improve again the safety of PQ (Heylings et al., 2007; Wilks et al., 2008; Wilks et al., 2011). Syngenta has increased the concentration of the emetic, added a purgative (magnesium sulphate), and included an alginate designed to cause gel formation of ingested product and thus delay absorption, namely by giving emetic enough time to work. Blood levels of PQ are indicative of outcome, but many patients have levels over 100 times the estimated lethal concentration, making treatment extremely difficult. In these patients, death may occur within a few hours from multi-organ failure, so measures to reduce PQ absorption seem a rightful choice. Nevertheless, there was some inconsistency in the approaches used to alter the formulation, in that gastric emptying was intended to be delayed by the use of an alginate that would gel in gastric acid. However, since emetics work predominantly after passive absorption from the small bowel, their absorption is necessarily coincident with the active uptake of the PQ in the small bowel. The alginate will thereby limit the

absorption of the emetic—hence the overall approach seems a little confused (Bateman, 2008).

The general aim of this thesis was to develop a safer PQ formulation with the incorporation of LAS as the antidote and preserving the herbicide effectiveness. Our research group has been exploring the effect of salicylates as antidotes for PQ poisonings. Previously it was clearly shown that NaSAL (200 mg/Kg i.p.) and LAS (200 mg/Kg i.p.) led to full survival of PQ intoxicated rats (Dinis-Oliveira et al., 2009; Dinis-Oliveira et al., 2007b). The major difference between NaSAL and LAS is the presence of an acetyl group attached to the phenol ring as in Aspirin® (ASA). In contrast to Aspirin®, LAS is a salt compound and therefore it is possible to dissolve LAS in the aqueous solution of PQ formulation. In humans, the plasma half-life of LAS is about 15 min, whereas for SAL it is between 2 and 30h, depending on the ingested dose. Because of the much longer half-life and an anti-inflammatory potency comparable to Aspirin®, it is probably salicylate that causes most, if not all, of the analgesic, antipyretic, anti-inflammatory, and antiproliferative actions of Aspirin® (as extensively explained in the review I in the theoretical background chapter). An exception is the antiplatelet effect that is specific for ASA and other targets that depend on the acetylation (Schrör, 2009a). For that reason, LAS constitutes an advantage since there are two bioactive components in one molecule. Although the mixing of PQ with an antidote appears to be a logical approach, there was no data so far showing that LAS or PQ would maintain its properties in a mixture. Besides this lack of data, as SAL forms a soluble crystalline charge-transfer complex with PQ (Dinis-Oliveira et al., 2008a), it was also important to verify the stability of the formulation concerning the herbicidal effectiviness and toxic effects. In Study I the safety of the formulation was evaluated using Wistar rats as a mammalian model. Diluted Gramoxone® (125 mg/kg) was mixed with increasing concentrations of LAS [half-molar (79mg/kg); equimolar (158mg/kg); and double-molar (316mg/kg) doses, relatively to PQ] and, for the first time, both compounds were administered by gavage. The mixture of PQ with LAS (125 mg/kg+316 mg/kg) proved to be safe, granting 100% of survival in comparison to only 40% observed in the PQ group. Noteworthy, in addition to the experiments with fresh solutions, experiments with two- and six- months-old formulations were also performed, and full survival was also achieved. Due to the commercial interest of this novel formulation, the herbicidal effect was also assessed against grass species belonging to the *Poaceae* family. Simultaneous foliar application of PQ and LAS provided similar efficacy to PQ alone when the solutions were freshly prepared. In the experiments carried out with two- and six-month-old formulations, the herbicidal activity was maintained, but the efficacy showed a time-dependent decrease. As mentioned above,

LAS is rapidly deacetylated to SAL in neutral or basic aqueous solutions which mean that after 2 or 6 months the formulation will mostly contain SAL and PQ. In view of the fact that SAL forms a soluble crystalline charge-transfer complex with PQ (Dinis-Oliveira et al., 2008a) it can be assumed that the decrease of the herbicidal efficacy is due to the formation of the complex PQ:SAL. Further work is necessary to assess the formulation stability, the full scale-up costs and the development of a technology able to prevent LAS hydrolysis within the formulation. In terms of the herbicidal effect the complexation is an inconvenience but regarding the mammalian toxicity it constitutes an advantage. Since 50% of LAS is immediately hydrolyzed in the gastrointestinal mucosa, and the remaining 50% is hydrolyzed within 15 min in plasma, PQ is either complexed before absorption (limiting the uptake by intestinal cells) or complexed with SAL after absorption within the plasma or cells. This putative mechanism of complexation with PQ *in vivo* might also contribute to the safer profile of LAS observed across all the studies of the thesis. Concerning the human toxicity, assuming a formulation containing PQ (200 g/L)+LAS (510 g/L), an ingestion of 30 mL would mean ingestion of 15 g of LAS. Acute life-threatening salicylates intoxication in adults occurs at doses of about 12–15 g and 3 g in children but under optimum conditions, mortality of severe intoxications amounts to less than 5% (Chyka et al., 2007). The treatment of salicylates poisoning include gastric lavage, administration of activated charcoal, and in cases of metabolic acidosis the administration of sodium bicarbonate (Schrör, 2009b). Given that the present formulation contains an emetic and that the measures mentioned for salicylates intoxication are also employed in cases of PQ poisoning, the concentration of both compounds will thereby be reduced. Besides, the PQ:SAL complexation should not be disregarded as it represents a valid pathway to reduce of the toxicity of both compounds. More importantly, the incorporation of LAS in PQ formulation will overcome the disadvantage of the critical time between intoxication and treatment, with an expected increase in survival rate and decrease in morbidity.

Nowadays, pesticides are widely used and may enter the aquatic ecosystems from agricultural runoff or leaching. The adverse effects of these pollutants on nontarget plants are particularly of concern due to the increasing worldwide use of these chemicals**.** Algae play an important role in the equilibrium of aquatic ecosystems, being the first level of the trophic chain to produce organics and oxygen. Accordingly, the subsequent objective of this thesis was to provide valuable information regarding the ecotoxicity of this new formulation using the microalga *Chlorella vulgaris* as a testing model. Our results corroborated previous studies showing that PQ significantly reduces the growth rate of microalgae cultures (Study II). Noteworthy, in the presence of LAS, the growth of *C.*

vulgaris is stimulated and is even higher than the control. Moreover, when PQ and LAS were added simultaneously, LAS exerted a beneficial effect. Indeed, LAS conferred protection against PQ toxicity of approximately 1.8 fold when the formulation was added in the proportion 1:8 in molarity.

The question remained: how is it possible to explain that this combination is non-toxic for *C. vulgaris* and at the same time still being effective against weeds? PQ herbicidal activity and algae toxicity is strongly associated with the inhibition of photosynthesis and the irreversible damage through the generation of ROS in a light-dependent manner. Despite higher plants and algae have the same target sites in photosynthesis, the differences rely on the formulation applied and its stability. In Study I we observed that LAS in the proportions 1:2 did not inhibit the herbicidal effect of PQ. Noteworthy, the solution was freshly prepared before application, but when the same study was performed with the 2 and 6 months-old a slight decrease in the efficacy was observed. Due to the neutral pH of the algae media, after 48h, almost all the LAS was hydrolyzed to SAL. Previous studies have shown that pre-treatment of seedlings with 0.5 mM SA for 24h before exposure to 10 µM PQ reduces PQ-induced chlorophyll losses, H_2O_2 production, LPO, electrolyte leakage, and completely blocked the inhibitory effect of the herbicide on photosynthesis (Ananieva et al., 2004). It seems that the rapid removal of SA by *C. vulgaris* plays an important role in the bioremoval and biotransformation of this compound since after 48h SAL was almost completely removed from the media.

Therefore, there are two possible mechanisms involved: 1) the SAL formed from the hydrolysis of LAS could complex with PQ thus preventing the inhibitory effect on photosynthesis, and 2) the antioxidant properties of LAS and SAL. In conclusion, in order to avoid the interference of SAL with the herbicidal effect of PQ, the formulation has to be applied immediately after preparation to overcome the instability problem with LAS. At the same time, the hydrolysis of LAS in the aquatic medium is advantageous to the environment as seen by the stimulation of algal growth in the presence of SA. Although the optimal effects were obtained at the ratio 1:8 (PQ:LAS), it is noteworthy that LAS, at the proposed ratio for the herbicide formulation of 1:2, was already protective for the toxicity mediated by PQ in the microalga *C. vulgaris*.

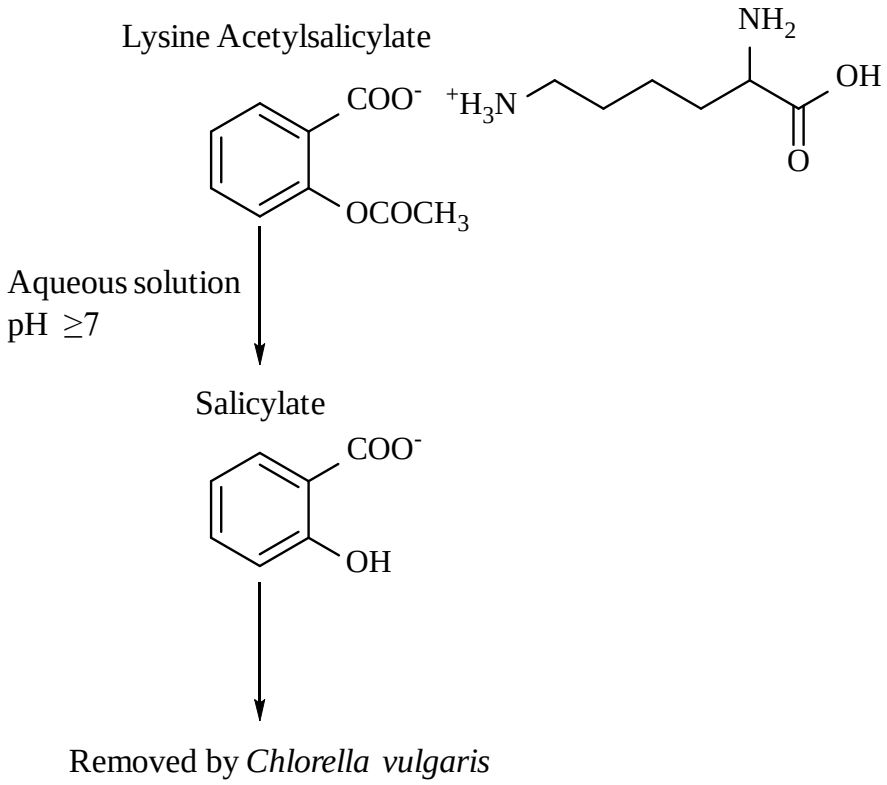


Figure 1. Metabolism of lysine acetylsalicylate (LAS) in the aqueous media and removal by *Chlorella vulgaris*.

Considering that the incorporation of LAS in the commercial formulation of PQ, Gramoxone®, was shown to significantly increase the survival of intoxicated mammals, while maintaining the herbicidal effect (Study I), the aim of the Study III was to clarify the mechanisms involved in the protective effect of LAS in a rodent model, through monitoring PQ levels and the histological and biochemical biomarkers of toxicity. The results clearly showed that LAS improves the safety of PQ formulation as shown by the much higher LD50 of the PQ+LAS formulation than the LD50 of Gramoxone® (34.86 mg/kg *vs*. 187.5 mg/kg, respectively). More specifically, LAS altered the toxicokinetics of PQ by increasing the PQ elimination in urine and feces, and decreasing plasma concentration. The results suggest that the alteration might be related to the improvement of the renal function, as sustained by the restoration of the clearance of creatinine and the levels of the lysosomal enzyme NAG, a tubular nephrotoxicity marker. LAS prevented the biochemical and histological alterations in lung induced by PQ at the end of 24h and 48h, showed by significant reduction in PQ-induced activation of NF-κB, reduction of infiltrative cells and preservation of the alveolar structure. This was evidenced by a significant reduction in LPO, the maintenance of GSH levels and decreased levels of GSSG in the lung. One may consider that the dose of LAS used in this study is quite high, yet the animals receiving

only LAS did not present any signs of toxicity. In mild to moderate salicylates poisoning, side effects such as nausea, vomiting, tinnitus, hyperventilation, respiratory alkalosis, tachypnea loss of coordination, and restlessness, can take place. Nevertheless, PQ poisoning is a serious life-threatening situation without an effective treatment and therefore the ratio benefit/risk will most probably tend to the benefits from the inclusion of LAS in the formulation of PQ. Though this new formulation has yet to prove to be less neurotoxic, because exposure to PQ may also be an etiological factor of PD and other neurodegenerative diseases (as extensively explained in the review II in the theoretical background chapter). Of note, only a single dose of LAS was given concomitantly with PQ to the animals. In hospital emergency rooms, first line therapy with fuller´s earth or activated charcoal, antioxidants and immunosuppressant agents are currently the measures adopted and the continuous infusion or repeated administrations of LAS are feasible, which could lead to a decrease of the subsequent doses of LAS.

In conclusion, the results obtained in the ambit of this thesis suggest that the new formulation developed is safer to mammalian and algae species with an effective herbicidal activity.

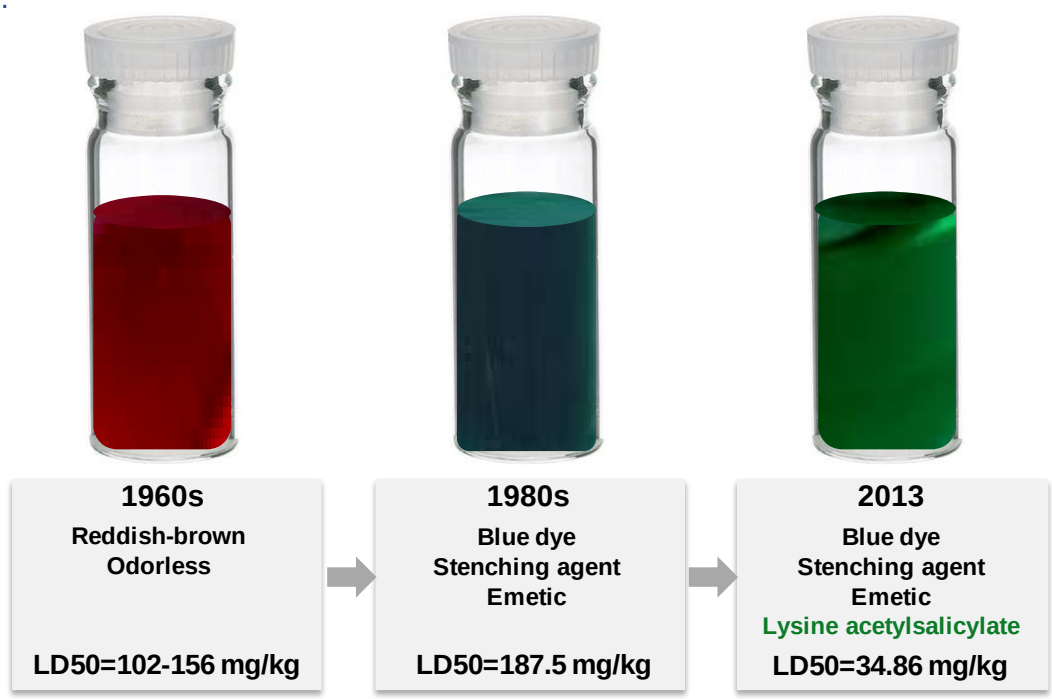
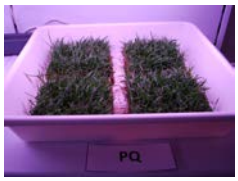
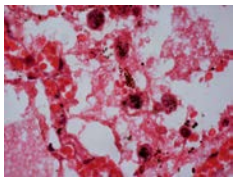


Figure 2. The evolution of paraquat formulations since its introduction in the market in 1962 until the present day.

CHAPTER VI

CONCLUSIONS

I. The administration of a new formulation containing PQ (125 mg/kg per os) and LAS (316 mg/kg *per os*) resulted in full survival of the animals (extended for more than 30 days) in comparison with the 40% of survival rate in animals exposed only to PQ.

II. The LD50 of the PQ+LAS formulation is higher than the LD50 of Gramoxone® (34.86 mg/kg vs. 187.5 mg/kg, respectively).

III. The foliar application of the new formulation containing PQ (2 g/L) and LAS (5.1 g/L) has a similar efficacy to PQ alone.

IV. There is a slight decline of the herbicidal activity of the formulations with 2- and 6-months of shelf storage.

V. Full survival was also recorded in animals treated with the formulations with 2- and 6-months old.

VI. *C. vulgaris* growth was almost completely inhibited by increasing concentrations of PQ. The values of EC50 and EC75 for PQ obtained were 525.5 µg/L and 1158.5 µg/L. The % of inhibition of growth was reduced in both groups PQ+LAS 1:1 and PQ+LAS 1:2 but the maximal effect was obtained for the proportion 1:8. In the end of the 96h, approximately 50% of the added LAS was depleted in the medium. The depletion of LAS was mainly due to its spontaneous hydrolysis into SAL in aqueous medium.

VII. SAL is, to some extent, biotransformed by *C. vulgaris* after 48 h and is not detected after 96h in the culture medium. The protection observed is mainly mediated by SAL.

VIII. PQ elimination in urine and feces was higher in animals treated with PQ+LAS than PQ only, at least 24h after exposure. After 48h, lung PQ concentration was lower in the group PQ+LAS than in PQ. The improvements of the elimination of PQ were accompanied by the restoration of the normal values of urinary creatinine, proteins, and NAG.

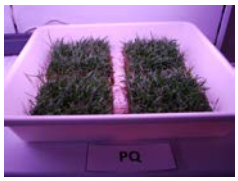
IX. The addition of LAS counteracted the PQ-induced ROS production and LPO in lung. MPO elevated values were not prevented by LAS but it was able to prevent the activation of NF-κB in lung.

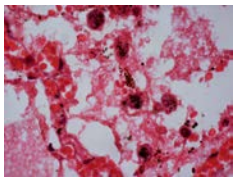
X. LAS prevented muscle toxicity, as shown by the normalization of the CK values. The hyperglycemic effect observed in PQ-treated rats was attenuated by LAS only in the PQ+LAS 48h group, probably due to a direct effect of LAS in lowering glucose levels rather that an amelioration of the pancreas toxicity since the amylase levels were unchanged.

XI. The overall aim of this thesis was accomplished since it was developed a safer formulation of PQ.

CHAPTER VII

FUTURE PERSPECTIVES

If PQ use is to continue, and we believe it will in many countries for the foreseeable future, then prospect projects are required to continue with pre-clinical studies in order to find the ideal combination of antidotes with the formulation. Due to the multiple mechanisms of PQ toxicity, salicylates seem to be the most complete antidote discovered so far, since they can block several signalling pathways involved in PQ toxicity. However, clinical data will be essential to assess the efficacy of salicylates towards PQ poisoning. Additionally, other pathways might also be important to be explored as the modulation of PQ toxicokinetics through the discovery of potent inducers/activators of P-glycoprotein or inhibitors of the uptake of PQ in the intestinal brush border and lung. Due to the differences of PQ levels found in the biological samples, the mechanisms underlying the influence of LAS in the uptake of PQ should be verified. These molecules together with antioxidants and anti-inflammatory drugs appear to be the right direction.

In relation to the formulation developed, further work is necessary to assess the formulation stability, the full scale-up costs and the development of a technology able to prevent LAS hydrolysis within the formulation. Additionally the effects of this new formulation on birds, fish, frogs, *Daphnia magna*, and other aquatic species should also be assessed. Future studies are necessary to verify the efficacy of this formulation on broadleaf weeds and in larger fields.

The sublethal effects such as respiratory symptoms, central nervous system disorders, dermal and inhalation toxicity of the new formulation should also be tested. Due to the global concern with the association of PQ exposure with neurodegenerative diseases such as PD, studies focusing in the evaluation of the protective effects of LAS or other drugs to the nigrostriatal system are also important.

CHAPTER VIII

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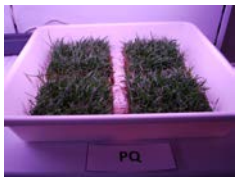


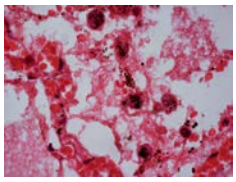

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