EFFECTS OF TUALANG HONEY ON PARAQUAT INTOXICATION IN RATS

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EFFECTS OF TUALANG HONEY ON PARAQUAT INTOXICATION IN RATS

by

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LIST OF SYMBOLS AND ABBREVIATIONS

%	percentage
˙OH	hydroxyl radical
µ	micro
A/G	albumin/globulin
AEAC	ascorbic acid equivalent antioxidant capacity
ALP	alkaline phosphatase
ALT	alanine transaminase
ANOVA	analysis of variance
A-P	anterior-posterior
APAP	acetaminophen
AST	aspartate transaminase
BG	blood glucose
BSA	bovine serum albumin
BUN	blood urea nitrogen
CAT	catalase
Cb	cerebellum
CC	cerebral cortex
CCl_4	carbon tetrachloride
CDC	Centers for Disease Control and Prevention
CDNB	1-chloro-2,4-dinitrobenzene
CoQ10	coenzyme Q10
CP	cerebral peduncle
DAB	3,3'-diaminobenzidine
ddH_2O	double distilled water
df	degrees of freedom
DOA	Department of Agriculture, Malaysia
DPPH	2,2,-diphenyl-1-picrylhydrazyl
DTNB	5,5’-dithio-*bis*-2-nitrobenzoic acid
DTPA	diethylenetriaminepentaacetic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FAMA	Federal Agricultural Marketing Authority
FR	Fenton reaction
FRAP	ferric reducing antioxidant power
g	gram
g	gravitational force
GGT	gamma-glutamyl transferase
GPx	glutathione peroxidase
GR	glutathione reductase
GSDNB	1-*S*-glutathionyl-2,4-dinitrobenzene
GSH	reduced glutathione
GSSG	glutathione disulfide (oxidized GSH)
GST	glutathione-S-transferase
H & E	haematoxylin and eosin
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid

HDL	high density lipoprotein
HMF	hydroxymethylfurfural
Hpc	hippocampus
HRP	horseradish peroxidase
Hth	hypothalamus
HWR	Haber-Weiss reaction
i.m.	intramuscular
i.p.	intraperitoneal
i.v.	intravenous
IC	inferior colliculus
IL-1β	interleukin-1 beta
IPCS	International Program on Chemical Safety
IQR	interquartile range
K_2HPO_4	potassium phosphate, dibasic
KCl	potassium chloride
KH_2PO_4	potassium dihydrogen phosphate
L	litre
LDL	low density lipoprotein
LPO	lipid peroxides
LV	left ventricle
m	milli
MDA	malondialdehyde
MES	2-(N-morpholino)ethanesulfonic acid
MO	medulla oblongata
MOH	Ministry of Health, Malaysia
MPA	metaphosphoric acid
MPP+	1-methyl-4-phenylpyridinium ion
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
Na_2HPO_4	sodium phosphate, dibasic
NaCl	sodium chloride
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NaH_2PO_4	sodium dihydrogen phosphate
NaOH	sodium hydroxide
nm	nanometer
O_2	oxygen
$O_2^{\cdot -}$	superoxide anion
OC	optic chiasm
OECD	Organization for Economic Co-operation and Development
ORAC	oxygen radicals absorbance capacity
p.o.	per os
Pb	lead
PBS	phosphate buffer saline
PFA	paraformaldehyde
PG	pituitary gland
PoA	preoptic area
PQ	paraquat
$PQ^{\cdot +}$	paraquat mono-cation radical
PQ^{2+}	paraquat dication
QH	ubiquinol

RA	right atrium
ROH	alcohols
ROOH	hydroperoxides
ROS	reactive oxygen species
s.c.	subcutaneous
SC	superior colliculus
SD	standard deviation
SEM	standard error of mean
SN	substantia nigra
SNpc	substantia nigra pars compacta
SOD	superoxide dismutase
SPSS	Statistical Package for the Social Sciences
TAC	total antioxidant capacity
TB	total bilirubin
TBA	thiobarbituric acid
TBS	Tris-buffered saline
TEAC	Trolox equivalent antioxidant capacity
TH	Tualang honey
Thal	thalamus
TMB	3,3′,5,5′-tetramethylbenzidine
TNB	5-thio-2-nitrobenzoic acid
TP	total protein
Tris-HCl	tris(hydroxymethyl)aminomethane-hydrochloride
Trolox	6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid
TyrH	tyrosine hydroxylase
v/v	volume per volume
w/v	weight per volume
WBC	white blood cells
WHO	World Health Organization
χ^2	chi-squared

KESAN MADU LEBAH TUALANG TERHADAP INTOKSIKASI PARAKUAT PADA TIKUS

ABSTRAK

Parakuat (PQ) adalah sejenis racun herba yang digunakan dengan meluas di seluruh dunia dan telah dipostulasi menunjukkan kesan toksik melalui penghasilan pelbagai spesies oksigen reaktif berikutan dengan kerosakan oksidatif pada komponen utama sel. Madu lebah Tualang (TH) dilaporkan mempunyai sifat antioksidan dan berkemungkinan membantu memperbaiki kerosakan oksidatif dalam kes keracunan PQ. Oleh itu, objektif utama kajian ini adalah untuk menilai kesan perlindungan madu TH pada ketoksikan akut (kajian 1) dan subakut PQ (kajian 2) di kalangan tikus. Tikus jantan Sprague-Dawley berusia lapan minggu digunakan. Pada kajian 1, dos oral PQ dan TH ditentukan pada 225 mg/kg dan 0.2 g/kg, masing-masing. Kesan rawatan TH tunggal dan berulang seterusnya dikaji: kumpulan rawatan tunggal menerima TH pada 0.5 (PQ + TH0.5h), 2 (PQ + TH2h) atau 6 (PQ + TH6h) jam selepas pemberian PQ; kumpulan rawatan berulang menerima TH pada 0.5, 2 dan 6 jam (PQ + THtrp) atau rawatan diteruskan sebanyak sekali sehari untuk enam hari berterusan (PQ + TH7d), masing-masing. Masa mandiri untuk setiap tikus dicatat sehingga hari ke-28 sebelum ia dikorbankan. Rawatan dengan TH tidak dapat menambah baik kadar kemandirian tikus yang diracunkan oleh PQ. Namun demikian, kumpulan tikus yang menerima rawatan berulang menunjukkan masa mandiri median yang lebih panjang secara signifikan berbanding dengan kumpulan PQ + TH6h. Rawatan dengan TH juga menambah baik hasil histologi pada tikus yang diracunkan dengan PQ terutamanya pada peparu yang mencadangkan bahawa kemungkinan kegunaan TH dapat melambatkan kesan toksik PQ. Pada kajian 2, kesan perlindungan TH pada tekanan oksidatif aruhan PQ pada otak tengah dan

peparu dikaji. Tikus dirawat dengan air suling (kumpulan N dan PQ; 2 mL/kg/hari), TH (kumpulan TH dan PQ + TH; 1.0 g/kg/hari) atau ubiquinol (PQ + QH; 0.2 g/kg/hari) setiap hari sepanjang masa kajian. Dua minggu selepas rawatan masing-masing, tikus diberikan larutan salin (N dan TH; 1 mL/kg/minggu, i.p) atau PQ (PQ, PQ + TH dan PQ + QH; 10 mg/kg/minggu, i.p) seminggu sekali selama empat minggu berterusan. Tikus dikorbankan seminggu selepas suntikan terakhir salin atau PQ. Hasil kajian menunjukkan bahawa kumpulan yang menerima TH (TH dan PQ + TH) atau ubiquinol (PQ + QH) mengandungi kandungan urea serum yang lebih rendah ($p < 0.05$). Kreatinina serum juga menurun secara signifikan di dalam kumpulan PQ + TH berbanding dengan kumpulan kawalan N dan TH. Kandungan ALT yang lebih rendah ($p < 0.05$) juga dilihat pada kumpulan TH dan PQ + TH apabila dibandingkan dengan kumpulan PQ. Perubahan ini mencadangkan bahawa rawatan TH mungkin memberikan kesan bermanfaat terhadap fungsi ginjal dan hati tikus. Berikutan pemberian PQ empat mingguan, aktiviti GPx otak tengah menurun secara signifikan. Kerosakan neuron dopaminergik akibat aruhan PQ dilihat apabila terdapat pengurangan signifikan bilangan neuron tirosina hidroksilase-imunopositif di kawasan *substantia nigra pars compacta* otak tengah. Penurunan signifikan aktiviti-aktiviti SOD dan GST di peparu juga diperhatikan pada kumpulan PQ berbanding dengan kumpulan N. Secara keseluruhan, rawatan TH dapat memperbaiki kesan-kesan toksik yang dilihat di otak tengah dan peparu, di mana kesan perlindungan TH adalah setanding dengan ubiquinol, ubat kawalan yang digunakan dalam kajian 2. Hasil kajian mencadangkan bahawa rawatan TH dapat memberi kesan perlindungan terhadap ketoksikan subakut PQ. Kesan ini mungkin berlaku akibat sifat antioksidan yang dimiliki TH.

EFFECTS OF TUALANG HONEY ON PARAQUAT INTOXICATION IN RATS

ABSTRACT

Paraquat (PQ) is an herbicide widely used in the world and has been postulated to exert its toxic effects via the production of various reactive oxygen species causing subsequent oxidative damage at key cellular components. Tualang honey (TH) has been reported to possess good antioxidant properties and may help ameliorate the oxidative damages in case of PQ poisoning. Therefore, the main objectives of this study were to evaluate the possible protective effect of TH in acute (study 1) and subacute (study 2) PQ toxicities in rats. Male Sprague-Dawley rats aged eight weeks old were used. In study 1, selected oral doses of PQ and TH were 225 mg/kg and 0.2 g/kg, respectively. The effects of single and multiple TH treatments on PQ-intoxicated rats were then investigated: single TH treatment groups received TH at 0.5 (PQ + TH0.5h), 2 (PQ + TH2h) or 6 (PQ + TH6h) hours following PQ administration; multiple TH treatment groups received TH at 0.5, 2 and 6 hours (PQ + THtrp) or further daily treatment for next six days (PQ + TH7d) following PQ administration, respectively (n = 6 per group). The survival time of each rat was recorded until day 28 before sacrifice. Although treatment with TH did not improve the survival rate of PQ-intoxicated rats, the median survival time of rats which received multiple TH treatments was significantly longer when compared to group PQ + TH6h. Furthermore, TH treatment improved the histological outcome of PQ-intoxicated rats particularly in the lungs, thus suggesting the potential role of honey in delaying the toxic effects of PQ. In study 2, the protective effect of TH on PQ-induced oxidative stress in rats' midbrain and lung regions were investigated (n= 15 per group). The rats were orally treated with distilled water (Groups N & PQ, 2

mL/kg/day), TH (Groups TH & PQ + TH, 1.0 g/kg/day) or ubiquinol (Group PQ + QH, 0.2 g/kg/day) throughout the experimental period. Two weeks after the respective treatments, the rats were administered with saline (Groups N & TH; 1 mL/kg/week, i.p) or PQ (10 mg/kg/week, i.p.; Groups PQ, PQ + TH and PQ + QH) once a week for four consecutive weeks. The animals were then sacrificed a week following the final injection of saline or PQ. Serum urea was significantly lower in groups which received TH (TH and PQ + TH) or ubiquinol (PQ + QH). Serum creatinine was markedly reduced in group PQ + TH as well, when compared to controls (N and TH). Significantly lower levels of ALT were observed in groups TH and PQ + TH when compared to group PQ. These findings suggest that TH treatment may have some beneficial effects on the kidney and liver's function. Following four-weekly PQ-administration, the midbrain GPx activity was significantly reduced when compared to healthy control (N). PQ-induced dopaminergic neuronal damage was demonstrated by a significant reduction in the number of tyrosine hydroxylase-immunopositive neurons in the midbrain substantia nigra pars compacta while in the lungs, marked reduction in the activities of SOD and GST were observed in group PQ when compared to the control group. Treatment with TH ameliorates the toxic effects seen in the midbrain and lungs with comparable effects to ubiquinol, the control drug used in study 2. These findings suggest that pre-treatment with TH showed some protective effects against subacute PQ toxicities. It is plausible that the protective effects of TH are conferred by its antioxidant properties.

CHAPTER 1

INTRODUCTION

1.1 RESEARCH BACKGROUND

Problems resulting from paraquat (PQ) exposure are reported all over the world and are mainly caused by suicidal intent, accidental poisoning or occupational exposures (Eddleston and Bateman, 2007; IPCS, 1991). PQ is known to be a producer of various reactive oxygen species (ROS) via single electron redox cycle *in vivo* (Abdollahi *et al.*, 2004; Autor *et al.*, 1977; Bus and Gibson, 1984). ROS readily attacks key cellular structures and molecules (lipids, carbohydrates, proteins and nucleotides) thus causing cellular deleterious effects which formed the basis of various disease conditions (Halliwell, 2009; Slater, 1984; Valko *et al.*, 2007).

In acute toxicity, high concentrations of PQ often lead to death due to multi-organ failures with major pneumotoxic effects seen (Bismuth *et al.*, 1990; Dinis-Oliveira *et al.*, 2007a; Fung *et al.*, 1999; Ghazi-Khansari *et al.*, 2005; Krieger, 2001). PQ is selectively concentrated in the lungs where the high alveolar oxygen content further increase the degree of lung injury by oxidative damage and exerts its major pneumotoxic effects in acute poisoning, thus producing diffuse alveolitis and loss of surfactant followed by progressive pulmonary fibrosis (Bismuth *et al.*, 1990; Yeh *et al.*, 2006). Similar effects may also occur in other major organ systems that eventually lead to death in the animals from multiple organ failure. In fact, ingestion of high doses of PQ usually leads to death from multi-organ failures. Smaller ingestion doses may also lead to delayed death as a result of extensive pulmonary

fibrosis and respiratory failure (Dinis-Oliveira *et al.*, 2006b; Suntres, 2002). Conventional approach in treatment of PQ poisoning focused on three main areas which include prevention of absorption from the gastrointestinal tract, enhancement of elimination of PQ from the body and administration of therapies directed against toxicity. Nevertheless, these treatment methods have been disappointing and the mortality rate remained very high (Eddleston and Bateman, 2007; Franzen *et al.*, 1991; Gawarammana and Buckley, 2011; Lin *et al.*, 1996).

On the other hand, unlike the major pneumotoxic effects occurring during acute poisoning, repeated low dose exposures to PQ is believed to be neurotoxic (Cicchetti *et al.*, 2005; Dinis-Oliveira *et al.*, 2006a; Franco *et al.*, 2010; Hatcher *et al.*, 2008). The effects of chronic exposure to PQ have gained considerable attention due to its wide usage around the world particularly among agricultural workers and PQ formulation workers (Cannon and Greenamyre, 2011; Drechsel and Patel, 2008; Tanner *et al.*, 2011). More alarmingly, there are growing evidences from epidemiological studies which implicated the possible involvement of environmental toxin such as pesticides as an aetiological factor for Parkinson's disease (Franco *et al.*, 2010; Hatcher *et al.*, 2008; McCormack *et al.*, 2002; Semchuk *et al.*, 1993).

Studies conducted by Hertzman *et al.* (1990) and Liou *et al.* (1997) indicated that exposure to PQ are associated with higher incidence of Parkinson's Disease. PQ-induced oxidative stress has been demonstrated in the substantia nigra region of Parkinson's disease brain (Franco *et al.*, 2010). A study by Ranjbar *et al.* (2002) reported that workers from PQ formulating factory are also at higher risk of having an oxidative stress as evidenced by elevated lipid peroxidation and decreased of

antioxidant power. In experimental models, rats or mice exposed to PQ demonstrate a selective degeneration of nigrostriatal dopaminergic neurons, one of the neuropathological hallmark of Parkinson's disease (Brooks *et al.*, 1999; Kang *et al.*, 2010; Kuter *et al.*, 2007; LeDoux, 2005; McCormack *et al.*, 2006).

Since the main suggested mechanism for PQ toxicity, either acute or chronic, involves the production of ROS, it may be hypothesized that an antidote against PQ poisoning should be a substance with good antioxidant properties. Honey is known to be rich in both enzymatic and non-enzymatic antioxidants. It contains both aqueous and lipophilic antioxidants with the interaction between these antioxidants suggesting its potential as an ideal natural antioxidant that can act at different cellular sites in the case of PQ poisoning (Angela, 2003; Küçük *et al.*, 2007; Nagai *et al.*, 2006).

Tualang honey is a wild honey harvested from the Tualang trees found in the Malaysia rain forest. Various studies have been conducted in an effort to evaluate the possible medicinal uses of Tualang honey including investigating its anti-cancer properties in cell culture and animal model (Fauzi *et al.*, 2011; Ghashm *et al.*, 2010), the protective effects of honey from cigarette smokes' damage (Mohamed *et al.*, 2011b), animal menopausal model (Zaid *et al.*, 2010), anti-diabetic properties (Erejuwa *et al.*, 2009; Erejuwa *et al.*, 2010a; Erejuwa *et al.*, 2010b) as well as in wound management and as an antimicrobial (Khoo *et al.*, 2010; Nasir *et al.*, 2010; Tan *et al.*, 2009). In addition, the neuroprotective effects of Tualang honey have also been previously reported in chronic cerebral hypoperfusion-induced neurodegeneration in the hippocampus of rats (Saxena *et al.*, 2014). Supplementation of Tualang honey improved the hippocampal and medial prefrontal cortex

morphology, memory performances and cholinergic system in stressed ovariectomised rats (Al-Rahbi *et al.*, 2014a; Al-Rahbi *et al.*, 2014b). One of the purported mechanisms of action is mainly contributed by its antioxidant properties.

A study by Mohamed *et al.* (2010) showed that Tualang honey contains phenolic compounds with good antioxidant activities. However, to date the protective effects of honey on PQ toxicity have not been investigated. Therefore, the main purpose of this study is to evaluate the possible protective effects of Tualang honey on PQ-induced acute toxicity (study 1) and subacute toxicity (study 2) in Sprague-Dawley rats.

1.2 OBJECTIVES

1.2.1 Study 1: The effects of Tualang honey on single-high dose exposure of paraquat in rats (acute toxicity study)

The general objective for study 1 is to evaluate the possible protective effect of Tualang honey in acute PQ poisoning. The specific objectives for study 1 include:

(i) To determine the dose of PQ that contributes to severe signs of toxicity following a week of its administration to rats.

(ii) To determine the dose of Tualang honey that can ameliorate PQ-intoxication in rats.

(iii) To determine the effects of single and multiple doses of Tualang honey treatments based on the survival rate at 28 days following PQ-intoxication in rats.

(iv) To determine the effects of single and multiple doses of Tualang honey treatments on the lung, kidney and liver's histological changes of PQ-intoxicated rats.

1.2.2 Study 2: The effects of Tualang honey on repeated-low dose exposures of paraquat in rats (subacute toxicity study)

The general objective for study 2 is to evaluate the possible protective effects of Tualang honey in the midbrain region and lungs of rats exposed to four weekly administration of PQ. The specific objectives for study 2 include:

(i) To determine the effects of Tualang honey on serum biochemical profiles of rats exposed to four weekly administration of PQ.

(ii) To determine the effects of Tualang honey on the oxidative stress parameters and tyrosine hydroxylase levels in the midbrain of rats exposed to four weekly administration of PQ.

(iii) To determine the effects of Tualang honey on immunohistochemical detection of tyrosine hydroxylase in the substantia nigra pars compacta region of rats exposed to four weekly administration of PQ.

(iv) To determine the effects of Tualang honey on oxidative stress parameters and histological changes in the lung of rats exposed to four weekly administration of PQ.

CHAPTER 2

LITERATURE REVIEW

2.1 PARAQUAT

2.1.1 History and uses

Paraquat (PQ) or 1,1'-dimethyl-4,4'-bipyridinium, is a synthetic quaternary ammonium compound first described by Weidel and Rosso in 1882. The redox poperties of PQ was discovered by Michaelis and Hill (1933) in 1933. In the early years PQ was commonly called methyl viologen and was used as a redox indicator. This is because PQ dication (PQ^{2+}) readily undergoes single electron reduction to form a stable free radical monocation ($PQ^{\cdot+}$) with blue or violet colours (Krieger, 2001). However, its herbicidal properties were only discovered in 1955 and finally it was first introduced to the market as a herbicide in 1962 by Imperial Chemical Industries Limited (ICI, now Syngenta). PQ is usually formulated as the dichloride salt. Although the patent protection on PQ has expired, Syngenta, one of the world's largest agrochemical companies, remains the major manufacturer for PQ under the trade name Gramoxone®, accounting for at least 50% of its total world market (Dinham, 2003; Wesseling *et al.*, 2001).

PQ is a non-selective, fast-acting contact herbicides applied for wide spectrum control of broad-leaved and grassy weeds, but have no effects on matured bark. It is used on over 100 crops in approximately 100 countries, some of which includes United States of America, China, Mexico, Thailand, Malaysia and Japan (Syngenta, 2015; Wesseling *et al.*, 2001). Its frequent application in a wide variety of plantation

crops have been reported to increase agricultural productivity in both developed and developing countries (Carlile, 2006; Centers for Disease Control and Prevention, 2003; IPCS, 1984).

2.1.2 Mode of action as herbicide

PQ exerts its herbicidal activity through the disruption of the normal electron flow in photosystem I which in turn inhibits the light reaction of photosynthesis. Once in the chloroplast where photosynthesis occurs, the positively charged PQ ion reacts with the free electron from photosystem I to produce PQ radicals. The radicals then react with oxygen to form various reactive oxygen species (ROS) which attack the unsaturated fatty acids of membranes, resulting in the destruction of membrane integrity and eventually cell death. Therefore, PQ is also known as cell membrane disruptors (Ashton and Crafts, 1981).

2.1.3 Paraquat toxicity

Despite its beneficial effects in agriculture, PQ is toxic to human and animals. Since its introduction in the early 1960's, fatalities due to intentional, accidental or occupational exposure to PQ have been frequently reported (IPCS, 1984; WHO, 2010). PQ is classified by the World Health Organization (WHO, 2010) as class II or moderately hazardous pesticide for acute toxicity based on its oral median lethal dose (LD_{50}) in rat (150 mg/kg). It is of relatively low hazard when used with adequate personal protective measures. However, serious delayed side effects may be developed which may even be fatal when the concentrated product is orally ingested or become in direct contact with the skin (WHO, 2010). The US Environmental Protection Agency (EPA) classified PQ dichloride into different categories of acute

toxicity based on the type of administration route i.e. highly toxic (category I) by inhalational route, moderately toxic (category II) by oral route, slightly toxic (category III) by dermal route; moderate to severe eye irritation (category II), and minimal dermal irritation (category IV) (Environmental Protection Agency, 1997).

2.2 TOXICOKINETICS OF PARAQUAT

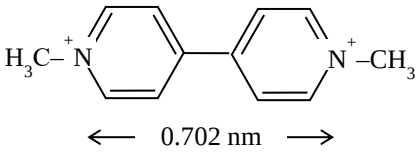
The intact skin is relatively impermeable to PQ but dermal absorption is enhanced when the skin is damaged (Wester *et al.,* 1984). Most cases of PQ poisonings however, results from oral ingestion. A rapid but incomplete absorption of ingested PQ occurs mainly from the small intestine. In human, it is estimated that only less than five per cent of the ingested amount reach the bloodstream over one to six hours following its ingestion (Houze *et al.*, 1990).

Regardless of its route of administration into the mammalian systems, PQ is rapidly distributed via the blood circulation to all organs and tissues of the body although its storage is not prolonged in any tissues, since it is being eliminated by the kidneys between days and weeks. The absorbed PQ is largely (> 90%) excreted as a parent compound through the kidneys within 24 hours if normal kidney function is retained. Disturbance of normal renal function following high ingestion doses may cause renal tubular necrosis which in turn affects the elimination-distribution and accumulation in other organs (Hawksworth *et al.,* 1981). Houze *et al.* (1990) described a bi-exponential plasma concentration-time curve, with the initial and late phases of elimination half-lives being five and 84 hours, respectively. Plasma PQ is then selectively concentrated in the lungs via an energy-dependent process due to its structural similarity with naturally-occurring polyamines (such as putrescine,

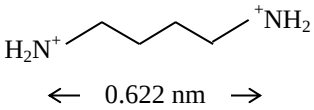
cadaverine, spermidine and spermine) following which it is taken up by the alveolar cells (Fung *et al.*, 1999) and exerts its major pneumotoxic effects. The structures of PQ and diamine putrescine are shown in Figure 2.1.

2.3 MECHANISM OF PARAQUAT TOXICITY

The main suggested mechanism for PQ toxicity is its ability to undergo cellular redox cycling with the subsequent production of various ROS (Bus and Gibson, 1984). Similar to its herbicidal mode of action, PQ^{2+} is readily reduced to mono-cation radical ($PQ^{\cdot+}$) by several enzymes including NADPH-cytochrome P450 reductase, xanthine oxidase, NADH-ubiquinone oxidoreductase (complex I of mitochondrial respiratory chain) and nitric oxide synthase (Day *et al.*, 1999; Fukushima *et al.*, 1993; Han *et al.*, 2006; Kitazawa *et al.*, 1991; Tawara *et al.*, 1996; Winterbourn and Button, 1984). $PQ^{\cdot+}$ is then rapidly re-oxidized to its original dicationic form in the presence of oxygen (electron acceptor). During the process, superoxide radical is generated at the expense of NADPH (electron donor). Further redox cycling of PQ leads to generation of other ROS including hydrogen peroxide and hydroxyl radical. Consequently, depletion of cellular NADPH may result in the disturbance of NADPH-requiring biochemical processes. Furthermore, ROS can induce oxidative damage to lipids, proteins and nucleic acids (Bus and Gibson, 1984; Dinis-Oliveira *et al.*, 2008; Lascano *et al.*, 2012). Schematic representation of mechanism of PQ toxicity is illustrated in Figure 2.2.



paraquat



putrescine

Figure 2.1 Structures of paraquat and putrescine showing the geometric standards of the distance between nitrogen (N) atoms. The substrate selectivity for polyamine uptake system includes two (or more) charged nitrogens, an ideal distance between the nitrogen and a nonpolar spacer to separate these charges (Boelsterli, 2007; Dinis-Oliveira *et al.*, 2008)

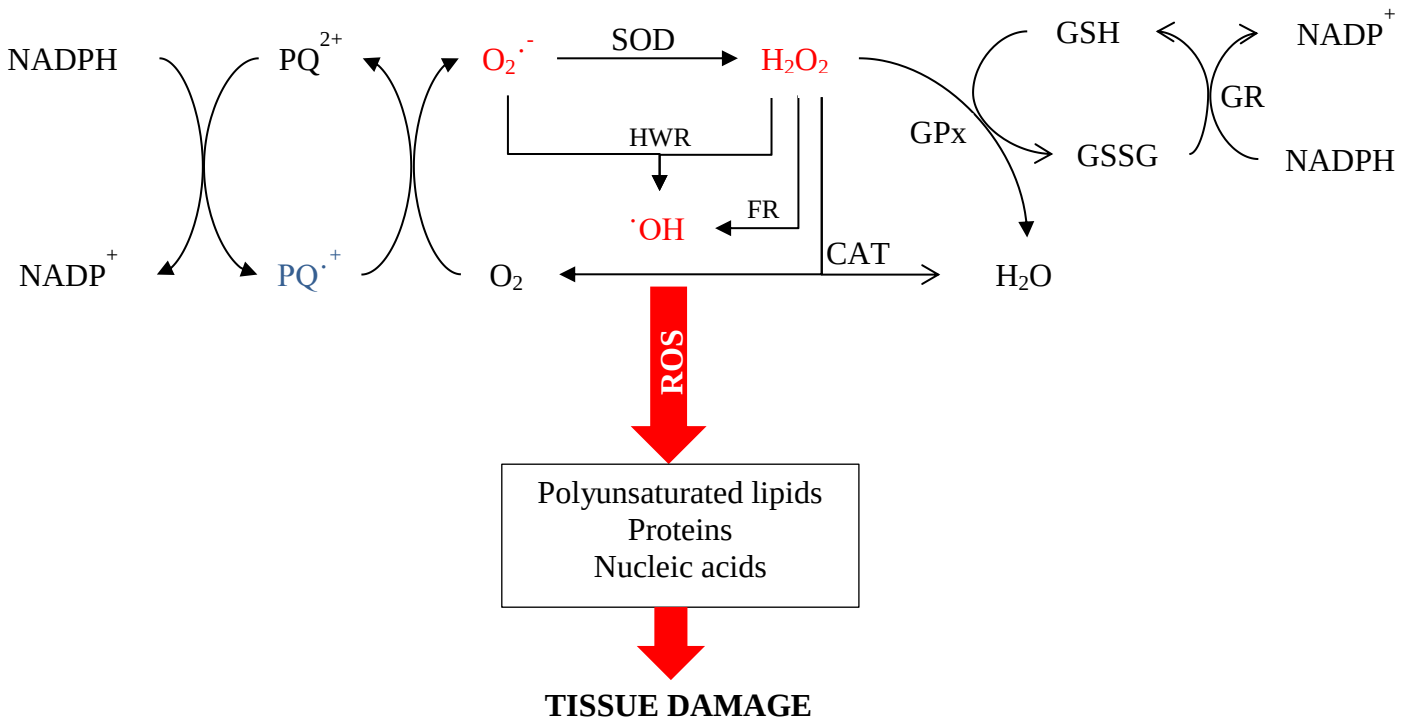


Figure 2.2 Schematic representation of the mechanism of paraquat toxicity. (Abbreviations: CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, glutathione disulfide; H_2O_2, hydrogen peroxide; HWR, Haber-Weiss reaction; FR, Fenton reaction; $NADP^+$, nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; O_2, oxygen; $O_2^{\cdot -}$, superoxide anion; $^{\cdot}OH$, hydroxyl radical; PQ^{2+}, paraquat; $PQ^{\cdot +}$, paraquat mono-cation radical; ROS, reactive oxygen species; SOD, superoxide dismutase) [Adapted from Dinis-Oliveira *et al.* (2008)].

PQ-induced generation of ROS and the subsequent oxidative stress reactions occur in most organs. However, the toxic effects of PQ are particularly severe in the lungs due to its selective accumulation through the polyamine uptake system. There are two distinct phases in the development of pulmonary fibrosis by PQ. In the early destructive phase, the alveolar types I and II epithelial cells are damaged, resulting in a second proliferative phase defined by alveolitis and finally leading to an extensive lung fibrosis (Bus and Gibson, 1984). Pulmonary damage is manifested by oedema, haemorrhage, infiltration of inflammatory cells into the interstitial and alveolar spaces and proliferation of bronchial epithelium. Eventually, individuals die of respiratory failure (Bus and Gibson, 1984; Dinis-Oliveira *et al.*, 2008; Ghazi-Khansari *et al.*, 2005; Smith *et al.*, 1990; Suntres, 2002) (Figure 2.3).

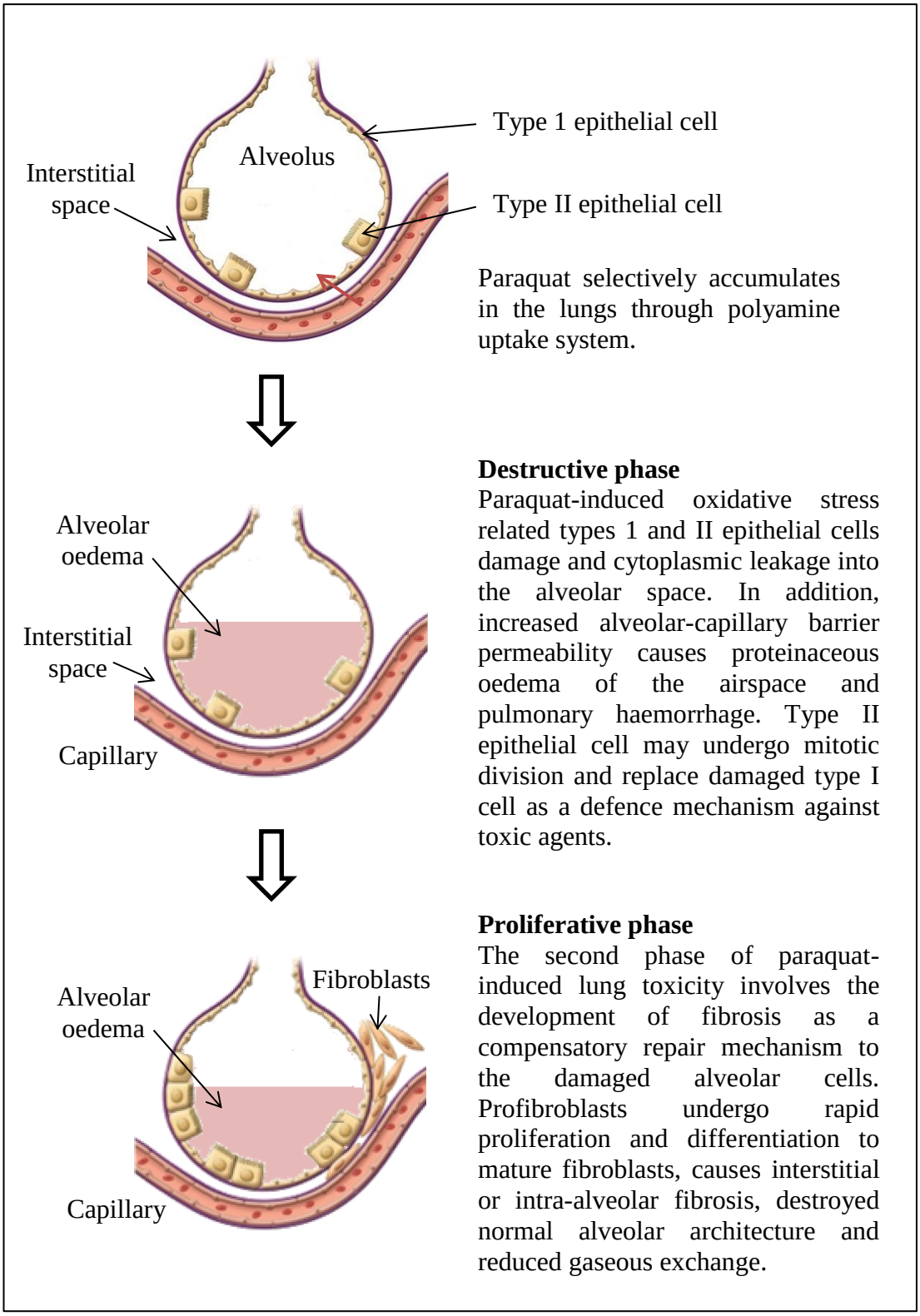


Figure 2.3 Pathogenesis of paraquat-induced lung toxicity.[Adapted from Dinis-Oliveira *et al.* (2008) and Matthay *et al.* (2012)]

2.4 ACUTE HEALTH EFFECTS OF PARAQUAT

Acute pesticide poisoning, often due to single-high level exposure, is one of the major health concerns of pesticide usage. Globally, suicides or intentional self-poisoning is considered as a major contributing factor for acute pesticide poisoning (Jeyaratnam, 1990). The estimated number of suicides in the year 2012 were 804 000 worldwide with 75.5% of the global suicides occurring in the low and middle income countries including Africa, Central America, South-East Asia and the Western Pacific regions (WHO, 2014). Pesticide self-poisoning is one of the three most common methods for suicide, accounting for approximately 30% of the global suicides, especially in the rural agricultural areas of low and middle income countries (WHO, 2014). Besides intentional poisonings, it has also been reported that an estimated 355,000 fatalities occurred due to unintentional poisonings worldwide (WHO, 2003).

Due to its popularity and ease of access, PQ is extensively used as a suicide agent particularly among developing countries (Gunnell and Eddleston, 2003; Jeyaratnam, 1990; Wesseling *et al.*, 2001). Nevertheless, a study by Seok *et al.* (2009) showed that only 38% of parasuicidal subjects intentionally select PQ suggesting that almost two-thirds of the subjects who ingested PQ occurred simply due to its wide availability during suicide attempts. On the other hand, accidental and occupational exposures to PQ were often associated with inappropriate use of pesticides, the lack of personal protective equipment or safety instructions on containers as well as accidental contamination with herbicide (Eddleston and Bateman, 2007; Eddleston *et al.*, 2002; Van Wendel de Joode *et al.*, 1996).

In Malaysia, almost three quarters (73.4%) of poisoning involving PQ were due to suicides, followed by accidental poisoning (13.8%) and occupational exposure (1.07%) (Jeyaratnam, 1990). Similar findings were reported between the year 2000 and 2006, where most chemical poisonings in Malaysia were parasuicidal, followed by accidental, homicidal and occupational-related poisonings where PQ is one of the main pesticides involved (MOH, 2006; Sirajuddin *et al.*, 2001). Poisoning (together with injury and other consequences of external causes) has been reported to be among the top ten reasons of hospitalisation and mortality in government hospitals in Malaysia (MOH, 2010; MOH, 2012). For instance, the review on the medical records of 79 PQ poisoning cases admitted to Hospital Taiping, Perak between January 2008 and October 2011 showed that 69.6% were of intentional exposure, followed by 26.6% accidental and 3.8% occupational exposures (Tan *et al.*, 2013).

Since pesticide poisoning accounts for nearly one-third of global suicides, it is suggested that death can be prevented if the use of toxic pesticides was restricted, or the access reduced through proper storage or disposal by individuals or communities. In addition, optimizing the medical management and the quality of care for poisoning cases are necessary (Gunnell *et al.*, 2007).

In an effort to reduce the accessibility of this highly toxic herbicide, beginning from August 2002, the Malaysian government has decided to ban PQ's use while all other previously registered PQ products would be phased out in stages by November 2007. However, the ban was lifted in November 2006 and registration of PQ was allowed for all crops (Krishnamoorthy, 2006). To date, 59 pesticides which contain PQ dichloride as an active ingredient are registered by the Pesticides Board, Department of Agriculture (DOA), Malaysia from February 2010 to January 2015 (DOA, 2015a).

Nevertheless, as of 2014, the use of herbicides containing PQ was restricted only to weed control in palm oil, rubber or dry paddy plantations as well as for killing of pineapple stumps only with its use forecasted to be banned again by the year 2020 (DOA, 2015b).

2.4.1 Clinical classification of acute paraquat poisoning

Clinical experiences of treating PQ-intoxicated patients help to predict the prognosis of PQ poisoning. Generally, the severity of PQ poisoning can be classified into three categories: mild, severe or acute fulminant poisoning. Patients who experienced mild poisoning, with an estimated ingestion of less than 20 mg PQ ion per kg body weight (or < 7.5 mL of 20% formulation for a 70 kg person), is often asymptomatic or may develop minor gastrointestinal symptoms such as vomiting and diarrhoea. However, in these cases, full recovery usually occurs (Vale *et al.*, 1987).

In cases of moderate to severe poisonings, with estimated ingestion of 20 to 40 mg PQ ion per kg body weight (or 7.5 – 15 mL of 20% formulation for a 70 kg person), patients may initially suffer from gastrointestinal symptoms, followed by multiple organs injuries. Proximal tubular dysfunction usually evolved and with renal insufficiency often noted, which may further affect the main route of PQ elimination and contribute to poor prognosis (Gil *et al.*, 2005). In addition, liver toxicity as revealed by associated biochemical abnormalities may also occur. Centrilobular hepatic necrosis and cholestasis are usually seen during postmortem examination of patients having impaired liver function (Dinis-Oliveira *et al.*, 2008). Death usually occurs in most cases. Nevertheless, patients who survived acute poisoning often developed pulmonary fibrosis, resulting in delayed death which usually occurs within

two to four weeks as a result of respiratory failure and is often fatal (Lock and Wilks, 2010; Vale *et al.*, 1987).

In cases of fulminant poisoning, with an estimated ingestion of more than 40 mg paraquat ion per kg body weight (or > 15 mL of 20% formulation for a 70 kg person), the mortality is almost 100%. Death usually occurs within the same day or no longer than a few days due to multiple organ failure where patients usually succumb before the development of pulmonary fibrosis (Reigart, 2009; Sabzghabaee *et al.*, 2010; Vale *et al.*, 1987).

2.4.2 Clinical management of paraquat poisoning

Clinically, there is no specific antidote for PQ poisoning. Athough a wide variety of therapies have been extensively studied, the mortality rate remains high with indeterminate treatment efficacies (Hong *et al.*, 2014; Nagami *et al.*, 2013). Therefore, the goal is to relieve symptoms and treat complications (i.e. to provide a supportive care). Conventional approaches in treating PQ poisoning include prevention of absorption from the gastrointestinal tract, enhanced elimination of PQ from the body and instituting therapy directed against toxicity symptomatically (Gawarammana and Buckley, 2011; Gil *et al.*, 2014).

The initial treatment of poisoning usually consists of administrations of oral absorbents such as Fuller's earth, activated charcoal or bentonite, all of which are aimed at neutralizing PQ in the gastrointestinal tract and enhancing its excretion in the faeces by using purgatives, mannitol or gastric lavage. For instance, routine treatment with multiple doses of activated charcoal also yielded no clinical benefit

with similar mortality rate compared to the non-charcoal treated groups (Eddleston *et al.*, 2008). Gastric lavage may be considered for patients who presented within an hour of ingestion of potentially life-threatening amount of PQ (Vale, 1997). However, serious complications which include hypoxia, gastrointestinal tract or pharynx perforation, disturbances of fluid and electrolytes balances may also occur (Benson *et al.*, 2013; Vale and Kulig, 2004).

Haemoperfusion, haemodialysis, haemofiltration and forced diuresis may be used to enhance excretion of systematically absorbed PQ or allow stabilization of haemodynamic status of patients with multiorgan failures (Gao *et al.*, 2015; Gawarammana and Buckley, 2011). A combined therapy with haemoperfusion and continuous venovenous haemofiltration may prolong the survival time and reduce the number of early deaths due to multiorgan failures, thus providing an opportunity for further treatment. However, the mortality rate remains high and patients eventually die from respiratory failure (Gao *et al.*, 2015; Koo *et al.*, 2002).

Other supportive therapies include surgical approaches (lung transplantation) and radiotherapy (to prevent fibroblast proliferation and ultimate interstitial fibrosis) to the diseased lungs although they have not been proven to be effective (Franzen *et al.*, 1991). There are some reports that pulse immunosuppression with the use of methylprednisolone and cyclophosphamide can help prevent lung fibrosis in PQ-intoxicated patients although further confirmation with clinical trials are still required (Koh *et al.*, 2014; Lin *et al.*, 2006; Lin *et al.*, 1996).

Current researches on PQ poisoning have also been directed towards the use of antioxidants, since PQ induces its toxic effect through oxidative stress-mediated mechanisms. For example, the use of superoxide dismutase (SOD) in PQ intoxication allows the conversion of highly toxic superoxide anions to potentially less toxic hydrogen peroxide and water. However, the finding from a study has demonstrated that administration of SOD by continuous intravenous infusion failed to improve PQ's toxic effects (Block, 1979). SOD also failed to protect against PQ poisoning in vitamin E-deficient animals, possibly because in the absence of vitamin E, peroxidative chain reactions are triggered and sustained by small amount of superoxide anion escaping detoxification by SOD (Block, 1979). Moreover, SOD cannot enter the target membrane or can hardly adhere to the targets due to its high molecular mass and charge (Ilizarov *et al*., 2001; Suntres, 2002).

Other antioxidants investigated in search of the treatment for PQ poisoning include vitamins C and E, melatonin, iron chelators, low molecular weight thiol-containing antioxidants [e.g. glutathione (GSH), N-acetylcysteine, metallothionein] and mono-unsaturated fatty acids. However, most of the antioxidants failed to modify PQ's toxicity which is attributed by their inability to cross cell membrane barriers and/or due to their rapid clearance from cells (Suntres, 2002). More recently, the use of liposomal antioxidants (e.g. liposome-entrapped GSH and α-tocopherol liposomes) or low molecular weight SOD mimetics leads to increased therapeutic potentials against PQ pulmonary toxicity since they presumably facilitate the intracellular delivery. Therefore, much remains to be known about the use of intracellular and extracellular antioxidants in PQ toxicity with no single strategy appearing to show

improvement in the outcome for PQ poisoning (Suntres, 2002) indicating that there is still a need to search for new treatment modalities.

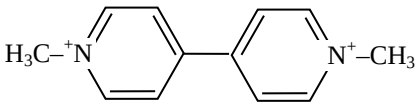
2.5 CHRONIC HEALTH EFFECTS OF PARAQUAT

In addition to the above, the possible delayed health effects from long term-low dose exposure is another major health concern of pesticide use particularly due to occupational exposure by the workers who were exposed to PQ for a long period of time. Additionally, chronic low dose exposures to PQ may also cause adverse respiratory effects among the workers. A study conducted among PQ workers in the Western Cape, South Africa indicated that under the usual field conditions, working with PQ over a long period of time is associated with desaturation of arterial oxygen, especially during exercise (Dalvie *et al.*, 1999). Castro-Gutierrez *et al.* (1997) reported an increase in the prevalence of respiratory symptoms among the workers. Analysis of blood samples from PQ-formulating factory workers have been reported to show increased in lipid peroxidation and decreased in antioxidant power (Ranjbar *et al.*, 2002).

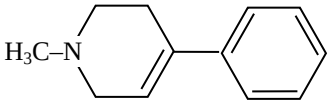
2.5.1 Paraquat neurotoxicity

In addition to its well-known pneumotoxic effects, the possible chronic neurotoxic effects of PQ have gained a wide interest over the past two decades due to its structural similarity with 1-methyl-4-phenylpyridinium ion (MPP^+) (Tieu, 2011; Wu *et al.*, 2012) (Figure 2.4). MPP^+ is an active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) with known dopaminergic neurotoxic effect contributing to acute Parkinsonism in human (Langston *et al.*, 1983; Shimizu *et al.*, 2003b). The by-product from synthetic heroin is not naturally found in the environment as

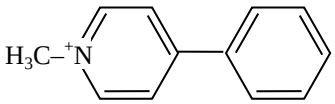
compared to the widely used herbicide PQ, further supported by human epidemiological studies which found that chronic exposure to pesticides including PQ, particularly among agricultural workers are associated with significantly higher incidence of Parkinson's disease (Hertzman *et al.*, 1990; Liou *et al.*, 1997; Petrovitch *et al.*, 2002).



Paraquat



MPTP



MPP^+

Figure 2.4 Structures of paraquat, MPTP and its active metabolite MPP^+. (Abbreviations: MPP+, 1-methyl-4-phenylpyridinium ion; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine).

2.5.2 Paraquat-induced oxidative stress-related neuronal damage

In an animal experimental model, a single PQ exposure did not induce any neurodegeneration in mice but can lead to microglial activation, making neurons more susceptible to damage following subsequent exposures (Purisai *et al.*, 2007). In an effort to understand the toxicokinetics of PQ in the brain, a study by Prasad *et al.* (2009) reported that PQ persists in the ventral midbrain with an estimated half-life of approximately 28 days, irrespective of its route of administration. The prolonged persistence suggests that accumulation of PQ following its repeated exposure is expected (Prasad *et al.*, 2009). Furthermore, systemic exposures of PQ in the rats or mice can induce selective dopaminergic neurodegeneration in the midbrain substantia nigra (SN) i.e. one of the neuropathological hallmarks of Parkinson's disease. In addition, dopamine depletion and increased levels of α-synuclein protein aggregation were also reported (Fernagut *et al.*, 2007; Manning-Boğ *et al.*, 2003; Wills *et al.*, 2012).

The brain naturally contains relatively low levels of antioxidants with high amounts of polyunsaturated fatty acids making it more susceptible to oxidative injuries due to redox imbalance. Of the brain's neuronal cell types, the dopaminergic neurons in the nigrostriatal system are selectively vulnerable to oxidative injury because dopamine metabolism itself can generate high levels of ROS (Chinta and Andersen, 2008). The role of oxidative stress in PQ-induced dopaminergic neurodegeneration in the SN region have previously been reported (Kang *et al.*, 2010; McCarthy *et al.*, 2004; Peng *et al.*, 2005; Somayajulu-Nitu *et al.*, 2009), thus suggesting the use of antioxidants as one of the possible therapeutic approaches for neurodegenerative disorders. For example, the study by Peng *et al.* (2005) demonstrated a protective role of synthetic

SOD/catalase mimetics in PQ-induced neurotoxicity in both rat dopaminergic cell lines and in adult mice. In addition, administrations of the antioxidant coenzyme Q_{10} (as known as ubiquinone) have also shown some neuroprotective effects in animal models of neurodegenerative disease (Cleren *et al.*, 2008; Matthews *et al.*, 1998; Somayajulu-Nitu *et al.*, 2009). Human preliminary data suggested daily supplementation of coenzyme Q_{10} appears to slow the disease progression in early Parkinson's disease (Shults *et al.*, 2002). Nevertheless, no evidence of clinical benefit was observed in a phase III clinical trial in patients diagnosed with Parkinson's disease within five years who received the coenzyme Q_{10} up to 16 months intervention and observation (Beal *et al.*, 2014), indicating its questionable effects.

Meanwhile, a recent published report on Phase II clinical trial using ubiquinol showed significant improvement in Parkinson's patient with wearing off (Yoritaka *et al.*, 2015). Ubiquinol, the reduced form of coenzyme Q_{10}, was previously showed to be more effective in an MPTP-induced mouse model of Parkinson's disease when compared to the same dose of coenzyme Q_{10} (oxidized form) tested (Cleren *et al.*, 2008). It is the most common form of coenzyme Q_{10} in vivo accounts for more than 80% of the total coenzyme Q_{10} (ubiquinol + ubiquinone) pool in human plasma, intestine and liver (Hosoe *et al.*, 2007). When administered orally, ubiquinol showed a higher bioavailability than the oxidized form of coenzyme Q_{10} (Mae *et al.*, 2001). However, the clinical efficacy for ubiquinol in Parkinson's patients was yet to be confirmed in a larger clinical study.