CHAPTER II
LITERATURE REVIEW

1. Parkinson's Disease

Parkinson's disease (PD) is a progressive neurodegenerative movement disorder that results primarily from the degeneration of dopaminergic neurons in the substantia nigra.

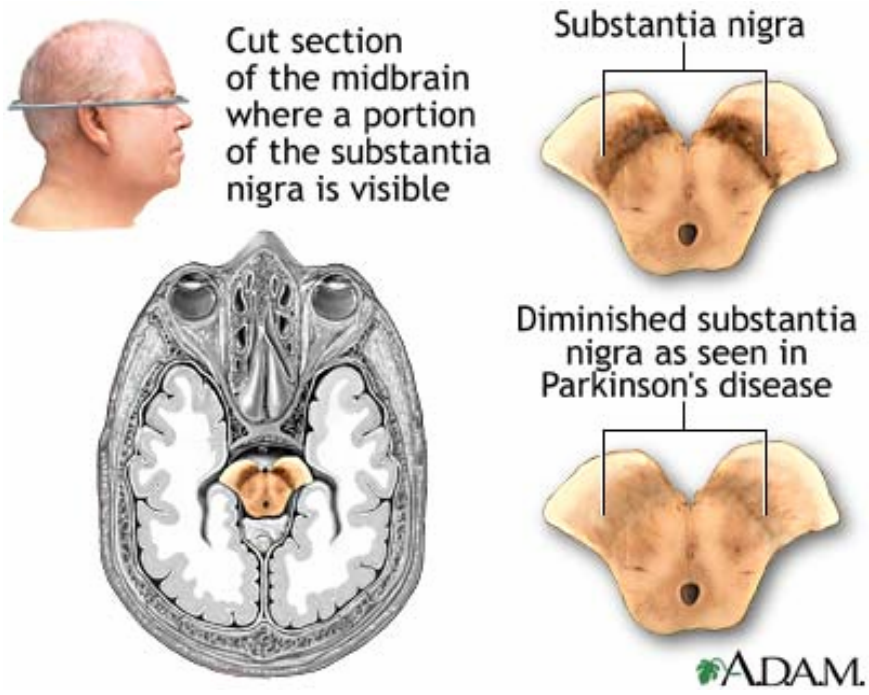

Figure 1 Substantia nigra and Parkinson's disease (Hoch, 2009).

1.1 The basal ganglia-thalamocortical circuitry

The major sources of input to the striatum are glutaminergic projections from the cerebral cortex and thalamus. Virtually, the entire cortex projects to the striatum in a topographic fashion. The cortex also projects directly to the subthalamic nucleus (STN). The substantia nigra pars compacta (SNpc) sends dopaminergic projections to the striatum. The striatum projects to the globus pallidus external segment (GPe), globus pallidus internal segment (GPi) and substantia nigra pars reticulate (SNr).

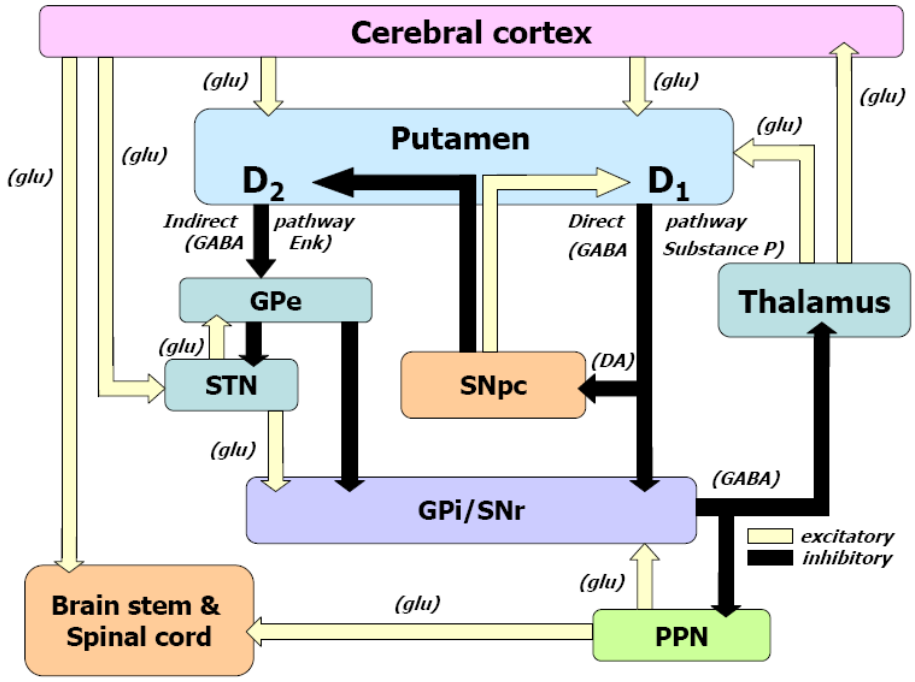

Figure 2 Motor circuit in Parkinson's disease (Hauser, 2010).

All outputs from the striatum utilize gamma amino butyric acid (GABA) but differ in the polypeptide cotransmitter. Striatal neurons projection to the GPe express enkephalin and possess predominamtly D_2 receptors. Whereas striatal neurons projecting to GPi express GABA and substance P and possess predominantly D_1 receptors. The GPe sends inhibitory GABAergic projections to the STN and GPi. The STN in turn sends excitatory glutamatergic projections to the Gpi and SNr and back to GPe. In addition, it was found that GPi sends GABAergic output to the ventrolateral (VL) and ventroanterior (VA) nuclei of the thalamus, which then send extensive glutaminergic projections back to the cerebral cortex. GPi also projects to the pedunculopontine nucleus (PPN) in the brainstem (Albin *et al.*, 1989; Nambu *et al.*, 1990).

In parkinsonian condition, the dopaminergic neurons in substantia nigra degenerated. Therefore, the activity of striatal neurons in the direct pathway is decreased resulting in a reduction of inhibition of GPi neurons, which in turn results in increased inhibition of the thalamus and a reduction of excitation of cerebral cortex, thus providing an explanation of loss and slowing of movement.

2. The Clinical Features of Parkinson's Disease

The clinical features of PD can be classified into two main groups including motor features and non-motor features.

2.1 Motor features

2.1.1 Bradykinesia

Bradykinesia or slowness of movement, is often used interchangeably with hypokinesia (poverty of movement) and akinesia (absence of movement). This symptom is the most commonly found symptom of basal ganglia dysfunction in PD (Jankovic *et al.*, 1999). In addition to the whole body slowness and impairment of fine motor movement, other manifestations of bradykinesia include drooling due to impairment of saliva swallowing of (Bagheri *et al.*, 1999). Previous study reported that the dopamine metabolism in the caudate nucleus was more affected in posture and direction of movement (Freed and Yamamoto, 1985). The study in PD patients pointed that bradykinesia occurred as a result of excessive activity in the subthalamic nucleus (STN) and the internal segment of the globus pallidus (GPi) (Dostrovsky *et al.*, 2002).

2.1.2 Tremor

Tremor at rest is one of the most recognized symptoms of PD (Hoehn and Yahr, 1967). It has been postulated that the typical resting tremor results from nigrostriatal degeneration and consequently inducing the disinhibition of the pacemaker cells in the thalamus (Findley and Gresty, 1984). These thalamic neurons discharge rhythmically at 5-6 Hz, a frequency similar to the typical parkinsonian resting tremor (Llinas and Jahnsen, 1982). The biochemical defect underlying of resting tremor is unknown. It was reported that the severity of tremor was paralleled with the degree of homovanillic acid reduction in the pallidum (Bernheimer *et al.*, 1973).

2.1.3 Rigidity and postural abnormalities

Rigidity may contribute to subjective stiffness and tightness, a common complaint in patients with PD. However, there is relative poor correlation between the sensory complaints experienced by most patients and the degree of rigidity (Koller, 1984; Snider *et al.*, 1976). In mild cases, cogwheel rigidity can be brought out by a passive rotation of the wrist or flexion-extension of the forearm

while the patient performs a repetitive voluntary movement in the contralateral arm (Matsumoto *et al.,* 1963). Rigidity may occur proximally (e.g., neck, shoulders, and hip) and distally (e.g., wrists and ankles). It is often associated with postural deformity, resulting in flexed neck and trunk posture and flexed elbows and knees, some patients develop ulnar deviation of hands (striatal hand), which can be confused with arthritis (Ashour *et al.*, 2005; Jankovic and Tintner, 2001). Other skeletal abnormalities include neck flexion (dropped head or bent spine) (Askmark *et al.*, 2001) and truncal flexion were also observed (Ashour *et al.*, 2005; Djaldetti *et al.*, 1999). The neurophysiologic mechanisms of rigidity are poorly understood. Spinal monosynaptic reflexes are usually normal in PD. It has been demonstrated that the degree of rigidity is correlated with the enhanced long-latency stretch reflexes (Berardelli *et al.*, 1983; Lee and Tatton, 1975; Rothwell *et al.*, 1983).

2.1.4 Postural instability

This impairment is important because it contributes to the fall-related injuries. The results from postencephalitic parkinsonism study revealed that globus pallidum degeneration was most responsible for the loss of righting reflexes and postural instability in pakinsonian patients (Purdon-Martin, 1967). It was reported that postural instability was correlated with reduced or absent vestibular responses (Traub *et al.*, 1980). However, the falls were still found in patients whose normal vestibular system (Koller *et al.*, 1989). This suggests that this impairment appears to involve another mechanism.

2.1.5 Freezing and other gait abnormalities

It was reported that the patients with PD usually exhibited a slow, shuffling, narrow-based gait (Jankovic *et al.*, 2001). The gait and postural problems associated with PD probably result from the combination of many factors such as bradykinesia, rigidity, loss of proprioceptive reaction, ataxia, vestibular dysfunction, and orthostatic hypotension. It was reported that one of the most disabling symptoms of PD is freezing or motor block, a form of akinesia (Giladi *et al.*, 1997; Giladi *et al.*, 2001). However, there are some tricks to overcome freezing, such as marching to command, visual cues as stepping over objects, walking to music or metronome, shifting body weight, and rocking movement (Dietz *et al.*, 1990; FitzGerald and Jankovic, 1989; Marchese *et al.*, 2000).

2.1.6 Other motor manifestations

These motor findings are directly related to one of the cardinal signs. The loss of facial expression (hypomimia, masked faces) and the bulbar symptoms (disarthria, hypophonia, disphagia, and sialorrhea) result from orofacial-laryngeal bradykinesia and rigidity (Hunker *et al.*, 1982). Respiratory difficulties occurred as a result from a restrictive movement due to rigid respiratory muscles and levodopa-induced respiratory dyskinesia (Jankovic and Nour, 1986; Rice *et al.*, 2002).

2.2 Non motor features

Besides motor features, patients attacked by Parkinson's disease also show other neurological symptoms which cause a considerable burden to the patient. Dementia is common and affects approximate 28% of PD patients (Aarsland *et al.*, 1996). However, the prevalence rate of dementia in this group of patient also varies depending on the age of the patient. It has been shown that the prevalence of dementia of PD patients over the age of 85 is increasing to 65% (Mayeux *et al*., 1990). Moreover, it was reported that dementia in PD patients seemed to show the rapidly progressive course (Mayeux *et al.*, 1988).

In addition to the dementia, other psychiatric symptoms also frequently coexist with Parkinson's disease especially depression (Mayeux *et al.*, 1981) and anxiety. Recent review has shown that depression is a common and potentially debilitating aspect of PD, affecting 40-50% of patients (Cummings and Masterman, 1999; Dooneief *et al.*, 1992; Zesiewicz *et al.*, 1999). It was also reported that depression in PD patients was often associated with anxiety and related to the disorder of serotonin transported allele (Menza *et al.*, 1999).

3. Neuropathology of Parkinson's Disease

3.1 Neurodegeneration

The essential pathological hallmark for the diagnosis of PD, is the loss of dopaminergic neurons (DA) in substantia nigra pars compacta (SNpc). It has been shown that DA loss in mild severity of PD patients is approximate 60%. However, it is not even all of the SNpc DA neurons that appear to be involved in PD. The ventral-lateral tier was more severely affected than the dorsal tier (Fearnley and Lees, 1991), and this accounted for a more severe loss of DA in the putamen particularly at dorsal

part which shows neurodegeneration as much as 95%, whereas those in caudate especially the ventral part showed neurodegeration only 60% (Kish *et al.*, 1988). However, it was found that, some central dopaminergic systems, such as the ventral tegmental area and hypothalamic system, were relatively spared, and descending spinal dopaminergic systems were spared entirely (Agid and Blin, 1987).

3.2 Lewy bodies

Another pathologic hallmark of PD is the Lewy body, an eosinophilic inclusion identified within neurons. According to the histological stains, Lewy bodies have an eosinophilic core, and a surrounding pale halo. They are usually rounded, although their shapes can be pleiomorphic (Gibb *et al.*, 1991), and they are generally 5 to 25 μm in diameter. They are usually existed within the soma, but can be seen in neurites or free in the extracellular space. Lewy bodies are commonly observed in the brain regions showing the most neuron loss in PD, including SN, locus coeruleus, the dorsal motor nucleus of the vagus, and the nucleus basalis of Meynert, but they are also observed in neocortex, diencephalon, spinal cord, and even peripheral autonomic ganglia (Gibb *et al.*, 1991). The ultrastructural analyses also demonstrated that Lewy bodies consisted of an electron dense granular core and a peripheral halo consisting of radially oriented filaments 7 to 8 nm in width (Duffy and Menefee, 1965). The filaments resemble neurofilaments, and can be immunostained with antisera to neurofilament proteins (Goldman *et al.*, 1983), including the NF-L, -M, and –H forms (Hill *et al.*, 1991). Another major antigenic feature of Lewy bodies is the expression of cellular proteins involved in protein degradation, including ubiquitin (Kuzuhara, 1998), and the proteasome (Fergusson *et al.*, 1996; Ii *et al.*, 1997). Presence of these antigens has been hypothesized to represent efforts on the part of the cell to degrade the abnormal protein aggregate. Moreover, another proteins particularly α-synuclein and syphilin were also observed in Lewy bodies (Baba *et al.*, 1998; Spillantini *et al.*, 1998; Spillantini *et al.*, 1997; Wakabayashi *et al.*, 2000).

4. Etiologic Factors

At present, the etiology of Parkinson's disease is not clearly understood. However, many factors have been proposed to contribute the roles in the genesis of PD.

4.1 Aging

The possible role of aging in the pathogenesis of PD is suggested by its usual occurrence in late middle age and by marked increases in its prevalence at older ages (Mayeux *et al.*, 1992). The possible contribution of age to the expression of the disease is further supported by early studies showing a loss with age of striatal DA (Carlsson and Winblad, 1976) and DA cells in the SN (McGeer *et al.*, 1977). Numerous studies have reported that there was the reduction of dopamine in striatum and neuronal loss in the SNpc with age but differ from what occurs in PD (Kish *et al.*, 1992; Scherman *et al.*, 1989). The loss of SN neurons in aging is linear and predominantly in the dorsal tier of the SNpc, whereas in PD it is exponential and predominantly in the lateral ventral tier (Fearnley and Lees, 1991; Scherman *et al.*, 1989). In addition, the SN in PD contains numerous reactive microglia, which are much less frequent in age matched control brains, indicating an active destructive process that is not present in the normal aged brain (McGeer *et al.*, 1988). Thus, whereas there is no question that increased age is a risk factor for PD, it remains unclear what precise role aging plays in pathogenesis.

4.2 Environmental factors

The role of environmental factors to induce PD has been focused since the Parkinsonism has been developed after neurotoxicity induced by MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a substance which has been sold as synthetic heroin (Langston and Ballard, 1983). Several toxins have been implicated in out break of parkinsonism, including carbon disulfide (Peters *et al.*, 1988), manganese (Mena *et al.*, 1967; Rodier, 1955), paraquat (Sechi *et al.*, 1992), and solvent abuse (Uitti *et al.*, 1994).

In addition to the drugs mentioned above, recent study also demonstrated that the organochlorine pesticide, dieldrin, also possessed the potential to induce neurotoxicity and Parkinsonism (Kanthasamy *et al.*, 2005). In addition, previous study also shown that rural residence, well-water drinking, or herbicide/pesticide exposure were recognized as the risk of developing PD (Tanner, 1992).

Numerous studies have attempted to detect occupational at high risk for developing PD. It was observed that there was a correlation between farming and PD (Gorell *et al.*, 1998; Liou *et al.*, 1997; Zorzon *et al.*, 2002). It was also observed that

orchard workers had a higher risk of PD (Hertzman *et al.*, 1990). The workers in the steel industry appeared to have high risk of PD because of the exposure to fumes which contain high amount of manganese (Cook *et al.*, 1974; Wang *et al.*, 1989; Whitlock *et al.*, 1966). There is some evidence that welders developed parkinsonism (Chandra *et al.*, 1981; Nelson *et al.*, 1993; Sjogren *et al.*, 1996; Whitlock *et al.*, 1966). Moreover, the blood sampling of welders showed high level of manganese (Chandra *et al.*, 1981) and aluminum (Sjogren *et al.*, 1996).

4.3 Genetic factors

Recently, it has been demonstrated that genetic factors appeared to be the predominant factors when PD developed before the age of 50. Data obtained from genetic study demonstrated that genetic variability in five genes including α−Synuclein, Parkin, Ubiquitin C-terminal hydrolase, DJ-1 and Tau were known to cause hereditary parkinsonism (Illarioshkin *et al.*, 2003).

α-synuclein is an abundant neuronal protein with an unknown function. It is the first protein implicated in familial PD. Although the precise role of this protein is still unknown, it has been proposed to contribute an important role in the pathogenesis of PD. It has been hypothesized that the pathogenesis of Parkinson's disease involves the abnormal folding, aggregation, and deposition of *a*-synuclein served as key steps in mediating neuronal dysfunction and degeneration (Feany, 2004).

The mutation in parkin gene was also reported. It is locating on chromosome 6, was first identified in Japanese families with a unique variant of parkinsonism (Kitada *et al.*, 1998). This form is inherited in an autosomal-recessive pattern, and typically begins at an early age (9-43 years) (Ishikawa and Tsuji, 1996). The immunohistochemistry study demonstrated that this protein localized in the cyroplasm of neurons at the regional level of SN and locus coeruleus (Shimura *et al.*, 1999). Parkin has been shown to play a role in protein degradation as a ubiquitin-protein ligase (Shimura *et al.*, 2000). These findings suggest that abnormal accumulation of proteins or abnormal regulation of the half-life of normal cellular proteins may play a role in cell death.

5. Pathogenic Mechanism

5.1 Free radicals and deficits in energy metabolism

The oxygen dependent (aerobic) respiration in the mitochondria is a big source of reactive oxygen species (ROS). The mitochondria generate energy in adenosine triphosphate (ATP) via Krebs cycle and electron transport chain (ETC). NADH and $FADH_2$, the high energy end products of Krebs cycle, donate electrons to a series of electron carriers on ETC which composed of complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), co-enzyme Q, complex III (cytochrome *bc*1 complex), cytochrome *c* and complex IV (cytochrome *c* oxidase). These components reduce molecule oxygen to H_2O_2. This transportation creates a proton gradient across the inner mitochondrial membrane which drives the oxidative phosphorylation of ADP to ATP by the ATP synthase. After passing through the series of electron carriers, the electrons donated by NADH and $FADH_2$ will eventually reduce oxygen molecules to water (Cadenas and Davies, 2000).

ROS are generated when electrons leak from ETC which results in a partial reduction of molecule oxygen to superoxide which mainly occurs at complex I (Lin and Beal, 2006; Olanow, 2007; Swerdlow *et al.*, 1996). Various enzymes can protect cellular components from oxidative damage, such as superoxide dismutase (SOD) which can metabolize superoxide to hydrogen peroxide (H_2O_2). Then, H_2O_2 can converted O_2 by catalase (Lin and Beal, 2006). These reactions are crucial for cells survival because superoxide and H_2O_2 can react with other molecules to generate additional ROS which can be extremely toxic to the cell. If H_2O_2 is not metabolized by catalase, it cab be converted to hydroxyl radical or hydroxyl anion in the present of ferrous iron through the Fenton's reaction (Chinta and Andersen, 2008; Winterbourn, 2008).

In addition to ROS, NOS also plays an important role on the pathogenesis of PD. Nitric oxide (NO) is generated by three isoforms of nitric oxide synthase (NOS); inducible NOS (iNOS), endothelial NOS (eNOS) and neuronal NOS (nNOS) (Kavya *et al.*, 2006). NO can react with other ROS to form the highly toxic RNS such as react with superoxide anions to form peroxynitrite that can further convert to highly intermediates such as nitrogen dioxide, carbonate and hydroxyl radicals (Reynolds *et al.*, 2007; Szabo *et al.*, 2007).

Recently, it was reported that NO exerted its influence on cell survival through S-nitrosylation, a reversible modification of cysteine (cys) residue in proteins to form the corresponding nitrosothiols (Ahern *et al.*, 2002; Hess *et al.*, 2005; Stamler *et al.*, 1992; Chung, 2007). It has been proposed that both ROS and RNS have been implicated in the degeneration of DAergic neurons (Danielson and Andersen, 2008).

Fahn and Cohen (Fahn and Cohen, 1992) have suggested that the free radical hypothesis was implicated due to four reasons. First, a major degradative pathway for DA is its oxidative deamination by monoamine oxidases A and B (MAO-A, MAO-B). This process results in the enzymatic production of H_2O_2, which, while itself not a free radical, can nevertheless react nonenzymatically with ferrous or cupric ions via Fenton-type reactions to form highly reactive hydroxyl radicals. Second, DA can react nonenzymatically with oxygen to form quinones and semiquinones, with the production of superoxide, hydrogen peroxide, and hydroxyl radicals. Third, the SN, particularly the SN pars reticulata, is rich in iron, which as mentioned above, may in its ferrous state catalyze the formation of hydroxyl radicals from H_2O_2. Fourth, the SN contains neuromelanin formed from the auto-oxidation of DA. This auto-oxidation generates toxic quinones and reactive oxygen species. In addition, the presence of neuromelanin in the cell may alter the ability of metal ions to participate in the production of reactive oxygen species (Swartz *et al.*, 1992).

Dexter and co-workers (Dexter *et al.*, 1989) have shown that in PD brain, there is a reduction in levels of polyunsaturated fatty acids, an index of the amount of substrate available for lipid peroxidation, and an increase in levels of malondialdehyde, an intermediate in the lipid peroxidation process. These workers subsequently confirmed evidence for abnormal lipid peroxidation in PD by identifying a tenfold increase in cholesterol lipid hydroperoxide, an early marker in the lipid peroxidation process (Dexter *et al.*, 1994). Postmortem studies have also revealed the neurochemical features that may predispose the PD brain to oxidative damage. Reduced glutathione is an important endogenous antioxidant, and it has been reported to be reduced in the SN in PD (Perry *et al.*, 1982). Jenner and colleagues have confirmed low levels of reduced glutathione in the SN of PD patients, and have shown that the alteration is disease-specific (Jenner *et al.*, 1992). A number of postmortem studies have also suggested that abnormalities of iron metabolism may

underlie the neurodegeneration of PD. Dexter and colleagues reported increased levels of iron in the SNpc of PD patients (Dexter *et al.*, 1989). This observation took on potentially greater significance when this group subsequently reported decreased levels of ferritin in PD brains (Dexter *et al.*, 1990), as ferritin normally sequesters iron in an unreactive state. However, it has become apparent that increased iron levels may be observed in many brain regions demonstrating neural degeneration in a variety of diseases of the basal ganglia (Dexter *et al.*, 1991), so the specificity of changes in iron levels in PD is less clear.

In addition to free radical theory, mitochondrial dysfunction has long been implicated in pathogenesis of PD. Such a defect could either result in the abnormal production of free radicals, or be the result of free radical injury (Schapira *et al.*, 1992). This hypothesis is linked with the group of drug abuse users who developed PD symptoms after accidental exposure of MPTP in 1980s (Langston *et al.*, 1983). MPTP is metabolized by MAO-B to MPP^+, a mitochondrial complex I inhibitor after it is uptaken by DAergic neurons results in the generation of excessive free radicals leading to neuronal death (Abou-Sleiman *et al.*, 2006). Inhibition of complex I can lead to the leakage of electrons from ETC and partial reduction of molecular oxygen to superoxide that can subsequently convert to other ROS (Cadenas and Davies, 2000). Mitochondrial dysfunction can contribute to the reduction of ATP biosynthesis leading to the inhibition of ATP dependent cytoprotective pathways such as 26S roteosomal mediated protein degradation, chaperone activities, and calcium pumps in the mitochondria and endoplasmic reticulum. Reduced ATP generation will also affect the transcriptional and translational processes that produce enzymes such as SOD and catalase to cope with oxidative damage (Schapira, 2008; Schapira *et al.*, 1989).

5.2 Protein aggregation

Based on the findings of α-synuclein in Lewy bodies of PD's brain, the possible role of protein aggregation as key factor responsible for the pathophysiology of PD had been suggested. Although the precise role of α-synuclein is unclearly known, it has been proposed that the aggregation of this substance might exert its toxic action by creating pores in lipid membranes (Volles and Lansbury, 2002) leading to the leakage of dopamine from vesicles into cytoplasm (Conway *et al.*,

2001). However, it was also reported that the aggregation of α-synuclein failed to show positive correlation with neuronal loss (Lo Bianco *et al.*, 2002). Thus the precise mechanism underlying the neurodegeneration induced by α-synuclein aggregation is still required further investigation.

ROS and RNS were known to produce protein misfolding and aggregation which occurred from the mutation of gene particularly α-synuclein (Abou-Sleiman *et al.*, 2006; Savitt *et al.*, 2006; Sulzer, 2007) in PD. In PD patient's brain, nitrated or oxidative damages protein aggregates are prominent (Danielson and Andersen, 2008). It is reported that nitrated α-synuclein is the major filaments building blocks of Lewy body (Giasson *et al.*, 2000). One toxic mechanism mediated by α-synuclein protofibrils occurred via the alteration of permeabilization of synaptic vesicle leading to the increase intracellular DA, which could further enhance DA modification of α-synuclein and consequently, the formation of more α-synuclein protofibrils (Mazzulli *et al.*, 2006; Mosharov *et al.*, 2006; Volles *et al.*, 2001).

Ubiquitin proteosomal system (UPS) or the lysosomal degradation pathways (autophagy) is a degrading system for damaged or misfolding proteins (Rubinsztein, 2006). Ubiquitin is a major component of Lewy body (Kuzuhara *et al.*, 1988). Parkin is an E3 ligase in the UPS and has been shown the neuroprotective effect on DAergic neurons against toxin insults (Shimura *et al.*, 2000). Thus, the oxidative stress could affect the protective function of parkin in PD (Winklhofer *et al.*, 2003).

5.3 Apoptosis

Apoptosis has long been implicated in the process of neurodegeneration in PD pathogenesis (Mattson, 2000). In addition, it was found that the programmed cell death of dopaminergic neurons also occurred in SNpc during the developmental period (Oo and Burke, 1997). Moreover, previous studies also demonstrated that this event was also observed even in animal models of parkinsonism. Intrastriatal injection of 6- OHDA produced an induction of apoptotic death in phenotypically defined DA neurons of the SN (Marti *et al.*, 1997). Previous finding indicated that caspase-3, apoptotic initiator was activated in Lewy body containing neurons in substantia nigra of PD's brain (Hartmann *et al.*, 2000). Unfortunately, the caspase-3 activation failed

to show the specificity. It was found even in control brain. Therefore, the role of programmed cell death in PD is still controversial.

5.4 Excitotoxicity

Overstimulation of N-methyl-D-aspartate receptors (NMDAR) by its ligand glutamate results in excitotoxicity (Calabrese *et al.*, 2007). This receptor is a ligand-gated Ca^{2+} channel. During the activation of NMDAR, the increased influx of Ca^{2+} leads to the activation of nNOS in a calmodulin-dependent manner (Dawson *et al.*, 1991). Activated nNOS produces NO which able to react with other ROS to form the highly toxic peroxynitrite, an important inducer to induce cellular damages through protein nitration, lipid peroxidation and DNA fragmentation (Szabo *et al.*, 2007).

Deregulation of cyclin-dependent kinase 5 (Cdk5) was also implicated to the excitoxicity induced PD (Qu *et al.*, 2007; Smith *et al.*, 2006). Cdk5 is a serine/threonine kinase that requires activating partners, p35 and p39 (Cheung *et al.*, 2006; Cheung and Ip, 2004). Its kinase activity plays an important role on many neuronal functions including development, migration, synaptic plasticity, axon guidance and neuronal survival (Cheng and Ip, 2003). The overstimulation of NMDAR has been shown to induce cleavage of p35 to p25, which is a stronger Cdk5 activator (O'Hare *et al.*, 2005; Wang *et al.*, 2007).

5.5 Inflammation

Available evidence suggests that the inflammation is an important mechanism in the pathogenesis of PD. It is well known that the function of glia is to control the homeostasis of the neuronal extracellular environment and respond to a diversity of damaging insults, including physical damage, disease or chemicals, which involve any cell type in the brain (Norton *et al.*, 1992). It has been demonstrated that there was glial reaction in the SNpc in PD which might be either protective or detrimental to dopaminergic neurons (Vila *et al.*, 2001). Microgia, the macrophage of the brain, is highly activated by PD (Banati *et al.*, 1998; Mirza *et al.*, 2000; Vila *et al.*, 2001). The important role of microglia is involved with the process of Lewy body formation which has three neurodegenerative phases; first concentric Lewy body-bearing neurons with no microglial involvement; second neurons with ill-defined, complement-positive Lewy bodies under intense microglial attack and third loose

extracellular Lewy bodies penetrated by astrocytic process (Togo *et al.*, 2001). In addition, astroglia and oligodendroglia were found near Lewy body-bearing neurons and the neurons die (Wakabayashi *et al.*, 2000).

In PD microglia in the SN have been shown to activated by macrophage marker EMB11 (Banati *et al.*, 1998), tumor necrosis factor-α (TNFα) (Boka *et al.*, 1994; Hunot *et al.*, 1999), interferon-γ (IF γ), interleukin-1β (IL1β), the low affinity immunoglobulin (Ig) E receptor CD23 (Hunot *et al.*, 1999), inducible nitric oxide synthase (iNOS) (Hunot *et al.*, 1996; Knott *et al.*, 2000), cyclooxygenase 2 *(*Knott *et al.*, 2000), complement 3 receptor (Banati *et al.*, 1998; Mirza *et al.*, 2000) and increased ferritin (Mirza *et al.*, 2000). These activated microglia cluster around DAergic neurons in an active mechanism of cell death call phagocytosis (Banati *et al.*, 1998; McGeer *et al.*, 1988; McGeer *et al.*, 1988). It was demonstrated that cytokines were also implicated with the observation of TNFα elevation in 6-OHDA-treated rats (Mogi *et al.*, 1999). In addition, there was the activation of microglia in SN and striatum following MPTP injection (Liberatore *et al.*, 1999). Therefore, cell death in PD relates directly to a substantial increase in microglia activation.

6. Animal Models of Parkinson's Disease

The incidence of neurodegenerative disorders in nonhuman animals is rare. However, the induction of specific phenotypes in a variety of animal species provides an important foundation to study neurological disorders and is important for determining the underlying disease mechanisms and developing new therapeutic modalities. In general, the utility of an animal model for a particular disease if often depend on how closely the model replicates all or part of the human condition. In PD, there is a variety of animal models have been derived from using multiple techniques. However, these models are not identical to the human condition with respect to behavioral characteristics, brain anatomy, or disease progression, but they have provided significant advancements in our understanding of the underlying mechanisms and treatment of PD. Recently, several toxin-induced, spontaneous, and transgenic models have been shown to display progressive motor deficits and/or degeneration.

6.1 MPTP

MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) is a neurotoxin used to mimic the Parkinsonian symptoms in animal models. At the beginning it is a side-product of the chemical synthesis of a meperidine analog with potent heroin-like properties. It is original from the inadvertent self-administration of MPTP by heroin addicts in the 1980s induced an acute form of parkinsonism whose clinical and biochemical features were indistinguishable from idiopathic PD (Davis *et al.*, 1979; Langston *et al.*, 1983). The rapidity with which these motor complications appeared presumably reflected the severity of substantia nigra neuronal degeneration induced by MPTP. Giving the similarities between the human model of MPTP-induced parkinsonism and PD, it became evident that MPTP could be used to develop animal model of PD.

MPTP-mediated toxicity is induced through conversion to the MPP^+ (1-methyl-4-phenyl-2,3-dihydropyridium ion) in astrocytes by monoamine oxidase B (Nicklas *et al.*, 1985). To exert its toxicity, MPP^+ must be transported into dopaminergic neurons by neurotransmitter transporters. Once inside the neuron, MPP^+ accumulates within mitochondria. Within mitochondria, MPP^+ acts by inhibiting the electron transport system of the mitochondrial complex I (NADPH-ubiquinone oxidoreductase I). This leads to the impairment of adenosine triphosphate (ATP) production, an elevation of the intracellular calcium concentration and the generation of free radicals, resulting in the failure of cellular energy (Nicklas *et al.*, 1987) and the formation of superoxide anions (Dawson, 2000) and degeneration of dopaminergic neurons. The selective toxicity of MPP^+ to dopaminergic neurons derives, at least in part, from its high affinity for the dopamine transporter (Javitch and Snyder 1984).

It was demonstrated that it has been widely used in nonhuman primates and mice (Schober, 2004). Unlikely PD, Lewy bodies have not been reported, however, eosinophilic inclusions (reminiscent of Lewy bodies) have been described in aged nonhuman primates (Forno *et al.*, 1986). The administration of MPTP to mice results in behavioral alterations that may resemble human parkinsonism. Whole body tremor and postural abnormalities have also been reported, but usually occurred in the acute phase (Sedelis *et al.*, 2001). Cognitive changes have also been reported with

respect to spatial memory (Tanila *et al.*, 1998). The MPTP-lesioned mouse model has been proven valuable to investigate potential mechanisms of neurotoxic-induced dopaminergic cell death such as mitochondrial dysfunction, energy depletion, free radical production, apoptosis and glutamate excitotoxicity (Royland and Langston, 1998). However, the study in rats has shown that MPTP injection did not exhibit any significant dopaminergic neurodegeneration (Giovanni *et al.*, 1994). This information is suggesting that MPTP may not be a suitable model for rats.

6.2 6-Hydroxydopamine

6-Hydroxydopamine (6-OHDA) or 2,4,5-trihydroxyphenylethylamine is a hydroxylated analogue of the natural neurotransmitter dopamine (Blunt *et al.*, 1991). The 6-OHDA induced rat model of PD was initially carried out by Ungerstedt in 1968, using stereotaxic bilateral intracerebral injections into the substantia nigra or lateral hypothalamus targeting the medial forebrain bundle (Ungerstedt, 1968).

6-OHDA induced toxicity is relatively selective for catecholaminergic neurons, resulting from a preferential uptake of 6-OHDA by dopamine and noradrenergic transporter molecules (Luthman *et al.*, 1989). The mechanism of 6-OHDA toxicity is complex and involves rapid auto-oxidation leading to the generation of hydrogen peroxide, superoxide and hydroxyl radicals and impairment of mitochondrial energy production (Blum *et al.*, 2001; Glinka *et al.*, 1997).

It has been reported that 6-OHDA can induce the apoptosis of dopaminergic cells in substantia nigra in rats which have been treated with 6-OHDA for 14 days (He *et al.*, 2000). However, previous study reported that inside neurons, 6-OHDA accumulates in the cytosol and induces cell death without apoptotic characteristics (Jeon *et al.*, 1995). It has been reported that 6-OHDA-induced neuron degeneration involves the processing of hydrogen peroxides and hydroxyl radicals in the presence of iron (Sachs and Jonsson, 1975). Furthermore, it has been shown that 6-OHDA treatment reduces striatal glutathione (GSH) and superoxide dismutase (SOD) enzyme activity (Perumal *et al.*, 1992) and increased the levels of malondialdehyde (Kumar *et al.*, 1995). 6-OHDA seems to be toxic to mitochondrial complex I (Betarbet *et al.*, 2002; Cleeter *et al.*, 1992) and leads to the formation of superoxide free radicals (Hasegawa *et al.*, 1990).

Systematically administered 6-OHDA fails to cross the blood-brain-barrier. Thus, 6-OHDA has to be injected stereotactically into the substantia nigra, medial forebrain bundle, and striatum (Perese *et al.*, 1989; Przedborski *et al.*, 1995). Following 6-OHDA injections into the substantia nigra or the medial forebrain bundle, dopaminergic neurons begin to degenerate within 12 h and striatal dopamine levels are depleted 2-3 days later (Faull and Laverty, 1969).

The early study using bilateral injection resulted in catalepsy, generalized inactivity, aphagia, adipsia, and a high degree of animal morbidity and mortality consequently the administration was modified to unilateral lesion which minimized postoperative morbidity, behavioral asymmetry, and a non-lesioned side to serve as control (Ungerstedt, 1971; Ungerstedt and Arbuthnott, 1970).

The advantage this model is that it is easy to assess the motor impairment by using rotational behavior (Ungerstedt and Arbuthnott, 1970). In general, a greater than 80% depletion of dopamine is necessary to manifest rotation in this model (Schultz, 1982; Schwarting and Huston, 1996).

Furthermore, intracerebral injection of 6-OHDA into the rat nigrostriatal pathway has been demonstrated to produce permanently degeneration virtually all dopaminergic neurons in the substantia nigra par compacta (Javoy *et al.*, 1976; Jeon *et al.*, 1995) leading to stable motor deficit over time. Previous studies have demonstrated that 6-OHDA lesioned rat model is an important tool in both elucidating the mechanism underlying motor complications (Cenci *et al.*, 2002; Monville *et al.*, 2005) and evaluating the pharmacological action of new drugs on the dopaminergic system, and the neuroplasticity following the nigrostriatal injury (Schwarting and Huston, 1996). In addition, this model has also been useful for determining important parameters for successful transplantation and the effectiveness of neurotrophic factors, antioxidant, the neuroprotective pharmacological agents and even the surgical techniques (Nikkhah *et al.*, 1994; Winkler *et al.*, 2000).

In summary, 6-OHDA-lesioned rat serves primarily as a model of dopamine dysfunction and highly specific for catecholaminergic neurons but it dose not replicate all of the behavioral, neurochemical, and pathological features of human PD. Overall, lesioning with 6-OHDA has provided a rich source of information regarding the consequences of precise dopamine depletion and its effects on rotational

behavior, dopamine biosynthesis, biochemical and morphological (lewy body) aspects of recovery. It was suggested that 6-OHDA was capable of inducing regional nigrostriatal degeneration in a topographical pattern very similar to idiopathic PD when injected into rodents (Rodriguez *et al.*, 2001).

6.3 Rotational behavior

This test has been first described by Ungerstedt and Arbuthnott since 1970. It is a quantitative assessment of rotational behavior following nigrostriatal damage in rats (Ungerstedt and Arbuthnott, 1970) which have to do after challenging apomorphine, a dopamine receptor agonist to stimulate the contralateral turning by the supersensitivity of D_1 and D_2 receptors preferentially on the lesioned side. Nevertheless, it was suggested that this phenomenon will occur until 90% loss of the dopamine afferent (Creese *et al.*, 1977). It was suggested that rotational behavior was a reliable test to determine the nigrostriatal damage (Ahmad *et al.*, 2005).

A distinctive behavioral feature of the unilateral lesioned model is rotation. This motor feature is due to asymmetry in dopaminergic neurotransmission between the lesioned and intact sides. Specifically, animals rotate to away from the side of greater dopaminergic activity. Pharmacologically induced rotation may be either contralateral or ipsilateral. Apomorphine, a dopamine receptor agonist induces contralateral rotation due to their direct action on super-sensitized dopaminergic receptors on the lesioned side. Conversely, d-amphetamine phenylisopropylamine induces ipsilateral rotation by blocking dopamine reuptake and increasing dopamine receptor activity on the non-lesioned side (Schwarting and Huston, 1996; Yuan *et al.*, 2005).

7. Quercetin

Quercetin (3, 5, 7, 3, 4-pentahydroxyflavone) is one of the most common natural flavonoids. Quercetin consists of two aromatic rings, A and B rings, and two rings are linked by an oxygen-containing heterocycle (ring C)

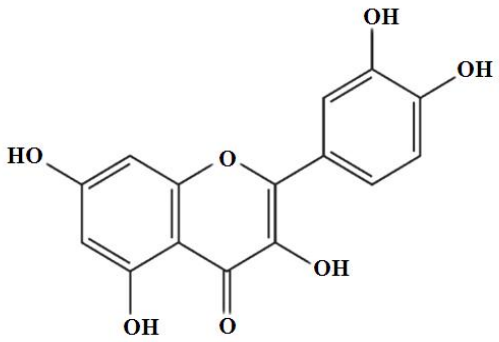

Figure 3 Structure of quercetin

7.1 Occurrence of quercetin

Human diets (berries, citrus, leafy vegetables, roots, tubers and bulbs, herbs, tea and cocoa) contain various amounts of flavonols (Brown, 1980). The study of Hertog and co-workers demonstrated that the highest quercetin concentration was found in onions (248-486 mg/kg), kale (110 mg/kg), French beans 32-45 mg/kg), broccoli (30 mg/kg), lettuce (14 mg/kg) and tomatoes (8 mg/kg) and among many fruits, apples have the highest quercetin concentration (21-72 mg/kg) (Hertog *et al*., 1992). The primary sources of natural dietary quercetin which presented as the aglycone in the Western diet are apples, cranberries, blueberries, and onions (Day *et al.*, 2000; Harnly *et al.*, 2006). Black tea and red wine have been identified as beverage rich quercetin (Hertog *et al*., 1993; Sampson *et al.*, 2002). The quercetin concentration was below 1 mg/litre in beverages includes beer, coffee, chocolate milk and white wine while quercetin in red wine was higher (4-16 mg/litre), grape juice contained 7-9 mg/litre, lemon juice contained 7 mg/litre, tomato juice contained 13 mg/litre, other fruite juices contained below 5 mg/litre (Hertog *et al*., 1993).

The estimated quercetin intake from daily diet was approximate 100 mg/day (Brown, 1980; Jones and Hughes, 1982; Rimm *et al.*, 1996; Sampson *et al.*, 2002). The mean quercetin consumption was only ~5-40 mg/day from various countries (Australia, the Netherlands, Finlands, Italy, Croatia, Japan, and the United States) (Hertog *et al.*, 1995; Johannot and Somerset, 2006; Kimira *et al.*, 1998; Knekt *et al.*, 1997; Lin and Beal, 2006; Rimm *et al.*, 1996). However, in those who consume

high amount of quercetin rich fruits and vegetables might have high quercetin level up to 200-500 mg (Jones and Hughes, 1982).

7.2 Biological properties of quercetin

Previous studies have demonstrated that quercetin exert the antioxidant effect (Chopra *et al.*, 2000; Hayek *et al.*, 1997), anti-carcinogenic (Deschner *et al.*, 1991; Pereira *et al.*, 1996; Verma *et al.*, 1988), anti-mutagenic (Geetha *et al.*, 2005), anti-inflammatory (Ferry *et al.*, 1996), and cardioprotective effects (Middleton *et al.*, 2000). It has been explained that the antioxidant effect of quercetin was related to the owing of hydroxyl groups (Rice-Evans *et al.*, 1996). It was reported that the structural features of effective antioxidant because of it contains an ortho-dihydroxy or catechol group in the B ring, a 2,3-double bond, and hydroxyl substitution at position 3 and 5 (Bors *et al*., 1990). In addition, several authors mentioned that its antioxidant of quercetin also involved with its hydroxylation position (3, 5, 7, 3' and 4' and a catechol B ring (Rietjens *et al.*, 2005; Silva *et al.*, 2002). Sun and co-workers have reported that quercetin exert the antiaging effect by producing significantly attenuate the spatial memory impairment in an animal model of aging induced by D-galactose (Sun *et al*., 2007).

In vitro studies, however, suggested that the oxidative degradation of quercetin might be involved with the production of a free radical orthosemiquinone intermediate, which may consequently involve with the formation of reactive oxygen species such as superoxide and hydrogen peroxide (Boots *et al.*, 2003; Metodiewa *et al.*, 1999). Therefore, it was suggested that quercetin might have the pro-oxidant activity in high dose (Rietjens *et al.*, 2005). Moreover, the early study using Ames test has revealed that quercetin was considered to be a carcinogen (Bjeldanes and Chang, 1977).

7.3 Absorption and metabolism

Quercetin is a flavonoid which consist of two benzene rings (ring A and B), which are connected by an oxygen-containing pyrone ring (C). It is commonly found in glycoside form, which has hydrophilic and high molecular weight which is the limitation to be absorption in the small intestine. In addition, the ß-glycoside also resists to the hydrolase enzymes in the intestine consequently unaltered quercetin can pass into the large intestine (Brown, 1980). It was demonstrated that the microflora of

the bowl produces glycosidase capable to release the aglycone from its sugar (Hawksworth *et al.*, 1971; Scheline, 1968). The cleavage of flavonoid ring also occurs in the large intestine (Baba *et al.*, 1981; Nakagawa *et al.*, 1965). However, the activity of glycosidase is faster than ring cleavage thus the intact flavonoid aglycone can exist in the large intestine with the potential to be absorbed. Therefore, it was believed that quercetin was absorbed only in the large intestine.

Nevertheless, following ingestion, quercetin will be metylated (3'-O-methylquercetin (isorhamnetin) and 4'-O-methylquercetin) then quercetin and its methyl derivatives will be conjugated to glucuronides or sulfates in the intestinal epithelium and absorbed here (Crespy *et al.*, 1999; Graf *et al.*, 2006; Murota *et al.*, 2000; Murota and Terao, 2005; Rechner *et al.*, 2002).

The study by using the [2-^{14}C] quercetin-4-glucoside in rats indicated that there was extensive intestinal metabolism but only very low amount (2.5%) transport across the gut wall into the bloodstream (Mullen *et al.*, 2002). A pipe of evidences have confirmed that quercetin and its metabolites were absorbed in the small intestine according to the representation of quercetin or quercetin conjugates was rapidly appear in plasma after consumption of foods containing quercetin glycosides (Boyle *et al.*, 2000; Carbonaro and Grant, 2005; Hollman *et al.*, 1995; Hollman *et al.*, 1997) and it was hypothesized that, the sodium-glucose cotransport (SGLT1) played a role on the transportation of quercetin glycosides across the enterocytes (Hollman *et al.*, 1999). It was suggested that the deglycosylation by enterocyte ß-glucosides occured after uptake (Gee *et al.*, 2000; Hollman *et al.*, 1997; Nemeth *et al.*, 2003). Day and co-workers have reported that the enzymes lactase-phloridzin hydrolase in the small intestine can hydrolyze the flavonoid glycosides (Day *et al.*, 2000). The results from perfusion studies revealed that at least of the formation of quercetin glucuronides and sulfates were taken placed in the intestinal wall (Crespy *et al.*, 1999; Spencer *et al.*, 1999).

The remaining quercetin derivatives and umetabolized quercetin from the small intestine will be released into the blood circulation via the hepatic portal vein. Then, quercetin and its derivatives will be further conjugated resulting in the formation of sulfates and/or glucuronides derivatives in the liver (Boersma *et al.*, 2002; Morand *et al.*, 1998; Oliveira and Watson, 2000; Shali *et al.*, 1991). In addition,

the catechol-O-methyltransferase (COMT) was also reported to play role on the further methylation of quercetin in the liver and kidney (De Santi *et al.*, 2002; Graf *et al.*, 2006; O'Leary *et al.*, 2003). The recently report suggested that quercetin could be systemically absorbed from the gastrointestinal tract via the lymph (Murota and Terao, 2005).

At the level of colon, the colonic microflora will degrade quercetin to one of several different phenolic acid such as 3,4-dihydroxyphenylactic acid and carbon dioxide (Booth *et al.*, 1956; Braune *et al.*, 2001; Chen *et al.*, 2005; Gross *et al.*, 1996; Krishnamurty *et al.*, 1970; Murray *et al.*, 1954; Olthof *et al.*, 2003; Pietta *et al.*, 1997; Rechner *et al.*, 2002; Stelzig and Ribeiro, 1972; Ueno *et al.*, 1983; Weldin *et al.*, 2003)

Many studies have demonstrated that the sulfate and glucuronide conjugated of quercetin and its derivative (O-methylate) were found in plasma following quercetin administration or another ways of consumption (quercetin rich foods or herbal extracts) (Ader *et al.*, 2000; Manach *et al.*, 1998; Manach *et al.*, 1997; Manach *et al.*, 1995; Manach *et al.*, 1999; Morand *et al.*, 1998; Morand *et al.*, 2000; Morand *et al.*, 2000; Morrice *et al.*, 2000; Zhu *et al.*, 1994).

Previous study in ACI rats has reported that only approximate 20% of radiolabeled quercetin was absorbed after a single oral dose (Ueno *et al.*, 1983). Recently, the absorption following an oral single dose (10 mg/kg BW) in Sprague-Dawley rat showed that total quercetin was found around 60% (free and conjugated quercetin, and its metabolites) and 5% was unchanged quercetin (Chen *et al.*, 2005). Justino and colleagues have reported that 93% of quercetin was metabolized following single intragastric administration of quercetin at dose of 50 mg/kg BW in Spraque-Dawley rats, the major identified metabolites were glucuronides, sulfoglucuronides, sulfates and isorhamnetin (Justino *et al.*, 2004). Following the quercetin rich diet in rats, the matylated quercetin such as isorhamnetin and tamarixetin were detected in plasma, urine and bile (Manach *et al*, 1996). Crespy and co-workers suggested that the main site of methylation in rats was in the liver, not the intestinal wall (Crespy *et al.*, 1999).

In healthy subjects, around 53% was absorbed following 100 mg of radiolabeled quercetin (Walle *et al.*, 2001). The studies in ileostomy patients have

demonstrated that the following the ingestion of quercetin 100 mg, 24 % of total quercetin was absorbed (Hollman *et al.*, 1995; Hollman *et al.*, 1997).

In human trial, the results showed that following the administration of quercetin rutinoside (rutin) and quercetin at dose of 50-65 mg/kg BW, there no unaltered form was observed in the urine (Kuhnau, 1976), or in plasma (Gugler *et al.*, 1975). However, the metylated quercetin was detected at the very low concentration in healthy volunteers after consumption of quercetin rich meal (Manach *et al.*, 1998).

The study of Petrakis and co-workers have demonstrated that following the administration for 12 hr, 44% of the radioactivity of 14C-labelled quercetin to adults rats was found in the intestinal tract particularly the lower bowl (44%), the remaining radioactivity was detected in the exhaled breath (15%), 12% was found in lung tissue, 3% in the wall of large intestine, less than 1% in blood, kidney and gastric wall, and only 4% in the urine but not as quercetin (Petrakis *et al.*, 1959).

The bioactivity of quercetin in vivo was suggested to involve with its metabolites in various tissues (glucuronated, sulfated and methylated). The unmetabolized quercetin was found in the GI contents for 94-100% whereas the quercetin in the GI tissues was present as many forms of sulfated, glucuronated and methylated, at 32% in stomach, 88% in small intestine, 27% in cecum, and 46% in colon. It was observed that quercetin was further metabolized postabsorption and found in liver, kidney, and plasma. The most observed form was sulfated methyl-quercetin (Graf *et al.*, 2006).

Following the administration of quercetin (lipophilic aglycone form) (30 μm/kg BW in different fat content; 1, 17 or 32 g fat/100 g) in growing pigs, the main metabolite found in plasma was always conjugated quercetin. The authors suggested that the fat content of the diet influences oral bioavailability of quercetin (Lesser *et al.*, 2004).

7.4 Bioavailability

Following the single or repeat quercetin administration at dose of 75-1000 mg (around 300-4000 mg/kg BW) in Wistar and Lister rats, the total plasma quercetin concentrations (free, conjugated quercetin and its metabolites) between 12.2-100 nmol/ml were detected (Carbonaro and Grant, 2005; Manach *et al.*, 1997; Manach *et al.*, 1999). Nagamura and colleagues have demonstrated that following repeated

giving quercetin at dose of 1 g/kg BW to male Wistar rats for 10 days, free quercetin was not detected, but unconjugated quercetin was found at the levels 0.56 nmol/mL (Nakamura *et al*., 2000).

However, free quercetin was detected at low concentration (0.9 μmol/L) following intragastric giving a single dose of quercetin (50 mg/kg BW) to male Spraque-Dawley rats (Justino *et al.*, 2004). In contrast, the quercetin aglycone was undetectable in plasma after intake 45% quercetin containing diet (~58.5 mg/day) of 6 weeks period of treatment in weanling rats (Graf *et al.*, 2006).

Study of plasma samples collected from volunteers following ingestion of single meal consisting of quercetin rich foods (~50 mg quercetin) demonstrated that quercetin levels (free, quercetin glycosides, glucuronides and sulphates) were 29-248 ng/mL (de Vries *et al.*, 1998; Hollman *et al.*, 1996; McAnlis *et al.*, 1999). However, the continuous daily ingestion of quercetin from onions at dose of 114 mg for a week showed the high level of total quercetin (453 ng/mL) (Janssen *et al.*, 1998).

In the earlier human trails failed to identify unconjugated quercetin aglycone following oral administration of flavonol (Erlund *et al.*, 2000; Graefe *et al.*, 2001; Manach *et al.*, 1998; Walle *et al.*, 2000). However, quercetin aglycone was identified in plasma after fried onion ingestion (Mullen *et al.*, 2004). The mean plasma quercetin levels was 8 ng/mL (120-350 ng/mL of total quercetin) in the subjects ingested quercetin 500 mg three times per day at a period of 5 days (Wang and Morris, 2005). Goldberg and co-workers have reported that the mean plasma aglycone level was 25 ng/mL after ingestion quercetin 10 mg/70 kg BW dissolved in beverages (vegetable juices, white wine and grape juice). It was noted that wine contains the highest level of quercetin (Goldberg *et al.*, 2003). Quercetin was previously thought not be absorbed in the study of Gugler and colleagues, because they failed to detect the quercetin in plasma and urine following 4 g orally administration in subjects (Gugler *et al.*, 1975).

7.5 Distribution

It has been reported that acute treatment with quercetin in Wistar rats showed the equal distribution of systematically-absorbed quercetin across all major tissues (Abrahamse *et al*., 2005). In the 11 weeks period of feeding quercetin in the diet, quercetin and its methylated derivatives especially isorhamnetin were identified

in various organs including lung, testes, kidney, thymus heart and liver. The conjugated forms of quercetin were primarily identified but free quercetin was also detected from lung, liver, kidney and testes at concentration up to 40% of total extracted quercetin (de Boer *et al.*, 2005).

7.6 Excretion

Since quercetin, conjugated quercetin and its metabolites were detected in plasma so it is believed that after quercetin was absorbed systematically, it may be eliminated in the urine (de Vries *et al.*, 1998; Gugler *et al.*, 1975; Ueno *et al.*, 1983; Wang *et al.*, 2003; Wang *et al.*, 2003; Young *et al.*, 1999) or may be secreted into the bile and excreted in the feces (Ueno *et al.*, 1983). In addition, quercetin was also degraded by microflora in the colon to phenolic acids which is eliminated in the feces (Gugler *et al.*, 1975; Ueno *et al.*, 1983) and carbon dioxide which is exhaled in the breath (Ueno *et al.*, 1983; Walle *et al.*, 2001); Abrahamse *et al*., 2005*)*. It was demonstrated that following oral administration in rats, the absorbed quercetin (~20% of administered dose) was excreted as glucuronide or sulfate conjugates via the feces (45%) and carbon dioxide in exhale breath (35%), and in urine (10%) (Ueno *et al.*, 1983).

While in the human trial, the major form of excretion following quercetin radiolabeled was carbon dioxide (41.8-63.9%), whereas in the urine and feces were only 3.3-5.7% and 0.21-4.6% respectively (Walle *et al.*, 2001). It was also observed that only small amount of parent form of quercetin was excreted in the urine within 8 hours after ingestion quercetin supplement at dose of 500 mg three times per day (Wang and Morris, 2005). Since the excretion also found in the bile so this indicated that the enterohepatic circulation appears to be the pathway that deconjugated quercetin was changed to aglycone in the lower intestine (Ueno *et al.*, 1983).

The results of previous studies suggested that the biliary elimination was the major route of quercetin elimination because they observed the concentration of quercetin and methylated metabolites in bile was higher than in urine. In addition, the quercetin glucuronides and sulfates were bound with protein (Boulton *et al.*, 1998; Manach *et al.*, 1995).

7.7 Toxicity

In the early studies, it was observed that quercetin exert the positive for mutagenic activity in *Salmonella typhimurium* (Bjeldanes and Chang, 1977; Cross *et al.*, 1996; Czeczot, 1994; Ueno *et al.*, 1984). DNA damage, chromosomal aberrations were observed following quercetin exposure (Caria *et al.*, 1995; Gaspar *et al.*, 1994; Kubiak and Rudek, 1990). In addition, the urine and fecal samples collected from rats administered quercetin orally demonstrated detectable levels of mutagenicity (Crebelli *et al.*, 1987; Stoewsand *et al.*, 1984). Interestingly, the methylatation of quercetin significantly attenuated its mutagenic activity (Brown and Dietrich, 1979; Czeczot *et al.*, 1990). In contrast, the plasma samples of rats received quercetin rich diet showed no mutagenic effect in Salmonella assays (Crebelli *et al.*, 1987).

Nevertheless, the mutagenicity of quercetin has not been showed in vivo studies. The administration of quercetin to rats and mice did not produce the alteration of parameters indicating the mutagenicity (Aeschbacher *et al.*, 1982; Cierniak *et al.*, 2004; Ishikawa *et al.*, 1985; Ngomuo and Jones, 1996; Taj and Nagarajan, 1996).

An early study indicated that LD50 of quercetin was in the range of 160 mg/kg BW (Sullivan *et al.*, 1951). However, following the single quercetin intravenous injection at dose of 100-150 mg/kg BW or double injection (up to 135 mg/kg BW) to the rabbits, no symptoms of toxicity were observed (Ambrose *et al.*, 1952).

In the toxicity study of Barrenetxe and co-workers have revealed that at a 28 days period of ~25 mg quercetin/day as 0.5% of the diet which corresponding to approximate 959 mg/kg BW/day to Swiss mice did not produce any significant changes in organ weights, biochemical parameters or intestinal histopathology (Barrenetxe *et al.*, 2006). This results were confirmed by a study of Ruiz and the results showed that quercetin at small amount to very high dose (30, 300, 3000 mg/kg BW/day) did not produce any changes of toxicity parameters (Ruiz *et al.*, 2006).

The proteinuria was observed in a 21 days feeding of high dose of quercetin (1133 mg/kg BW/day) in the hypertensive rats (Rangan *et al.*, 2002). The variations of fecal bile acid was reported in Wistar rats which received quercetin in diet for 22 days at dose levels up to 1000 mg/kg BW/day (Nakamura *et al.*, 2000). Following orally

quercetin administration at dose of 10 mg/kg BW for 5 weeks in hypertensive experiment in rats, there was no significant change of body weight, heart weights and kidney weight (Garcia-Saura *et al.*, 2005). Long term oral administration induced carcinogenicity was an important issue that has been concerned. Luckily, numerous studies have demonstrated that quercetin did not produce the neoplasm formation (Ambrose *et al.*, 1952; Hirono *et al.*, 1981; Hosaka and Hirono, 1981; Ito *et al.*, 1989; Morino *et al.*, 1982; Saito *et al.*, 1980; Stoewsand *et al.*, 1984). However, at the 59 weeks period (Pamukcu *et al.*, 1980), and 2 years of quercetin treatment was noted to relate with carcinogenicity (Dunnick and Hailey, 1992).

However, Okamoto concluded that quercetin administration via oral route is unlikely to cause any adverse effects (Okamoto, 2005). It has been reported by the early study that quercetin at dose of 300 mg/kg BW did not produce any worse effects on fertility in male rats or prevent the embryonic implantation in female rats (Aravindakshan *et al.*, 1985).

8. Transdermal Route Administration

During the recent years, transdermal application appears to be the interest alternative choice because patients often forget to take medicine, getting tried of swallowing pills and the long time of taking pills. Additionally, bypassing the gastrointestinal tract would obviate the irritation and avoid partial first-pass inactivation by the liver. In addition, transdermal application provides the steady absorption of drugs over hours or day leading to the therapeutic level. Due to its barrier properties, the skin membrane is equally capable at limiting the molecular transport from and into the body. Therefore, overcoming this barrier is the main purpose of transdermal drug delivery.

The goal of transdermal administration is usually designed to offer a slow, sustained release of drug over long periods of time. Current transdermal occlusive patches are capable to deliver drugs in cases of that oral administration is limited by poor bioavailability, side effects associated with high peak plasma concentrations or poor compliance due to the need of frequent administration (Naik *et al.*, 2000; Thomas and Finnin, 2004).

An essential physicochemical property of drugs will be able to develop for passive transdermal delivery. It should be aqueous solubility 1 mg/ml, lipophilicity 10<Ko/w<1000 (oil-water partition coefficient), molecular weight less than 500 Da, melting point less than 200 °C, pH of saturated aqueous solution pH 5-9, and the dose deliverable less than 10 mg/day (Naik *et al.*, 2000).

Under the normal conditions, there are three pathways postulated for the absorption of substances through the stratum corneum; transcellular, intercellular (paracellular) and transappendageal (Roberts and Cross, 2002). The predominant route of transdermal penetration is through intercellular spaces. Therefore, the drug must posses both lipoidal and aqueous solubility, which promote its permeation through the domains of the stratum corneum, the drug mobility must be high i.e. molecular weight and volume must be appropriate to facilitate its diffusion through the lipid bilayer. The permeation through the skin will also depend on the ionization degree of the drug at physiological and formulation pH, influencing as well as its solubility and partition behavior (Alvarez-Roman *et al.*, 2004; Cevc, 2004; Hadgraft, 2004; Naik *et al.*, 2000). In addition, the good drug should provide the enough amount of drug to overcome the skin barrier, no irritating effect on the skin, and drug will not be inactivated on the skin's surface or during the permeation process (Langer, 2004).

Transdermal absorption occurs through a slow process of diffusion driven by the gradient between the high concentration in the delivery system and the zero concentration prevailing in the skin. Thus, the delivery system must be kept in continuously contact with the skin for a considerable time. The criteria that merit consideration in transdermal delivery of drugs are: the nature of the barrier (skin), the balance between physicochemical properties of the membrane and the drug, and the technology available to facilitate the transdermal transport.

Transdermal drug absorption can significantly alter drug kinetics and depends on a variety of factors including site of application, thickness and integrity of the SC, size of molecule, permeability of the membrane of the transdermal drug delivery system, state of skin hydration, pH of the drug, drug metabolism by skin flora, lipid solubility, depot of drug in skin, and alteration of blood flow in the skin by additives and body temperature (Berner and John, 1994; Singh and Singh, 1993; Stevenson *et al.*, 1993).

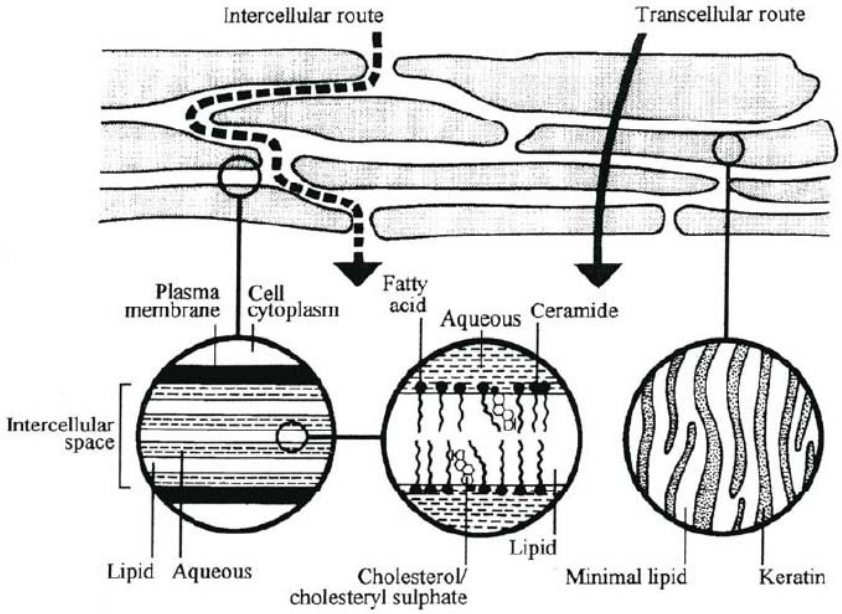

Figure 4 Schematic representation of the "brick and motar" model of the stratum corneum, lipid bilayer organization and possible pathways (Moghimi *et al*., 1996).

9. Skin

Skin (cutis) is considered the largest organ of the body, is divided into several layers but the outermost layer called stratum corneum (SC) is the best barrier of transdermal drug delivery. This layer is a compositionally and morphologically unique bio-membrane (Scheuplein and Blank, 1971). This structure has been described as brick and mortar model (Figure 4) and is considered the rate controlling barrier in the transdermal absorption of substance (Washington *et al*., 2001). It has an approximately thickness of 10-20 μm. It consists, in a given cross-section, of 15-25 flattened, stacked, hexagonal, and cornified cells (corneocytes, also called horny cells) anchored in a mortar of highly organized intercellular lipids (Christophers, 1971; Elias, 1983; Elias, 1981).

The lipid of this area are distinctive in many respects: (i) they provide the only continuous phase (diffusion pathway) from the skin surface to the base of the SC; (ii) its composition (mainly ceramides, free fatty acids and cholesterol) is unique among biological membranes and particularly noteworthy is the absence of phospholipids;

(iii) despite this deficit of polar bilayer-forming lipids, the SC lipids exist as multilamellar sheets; and (iv) the predominantly saturated, long-chain hydrocarbon tails facilitate a highly ordered, interdigitated configuration and the formation of gel-phase membrane domains as opposed to the more usual (and more fluid and permeable) liquid crystalline membrane systems (Gray *et al.*, 1982; Williams and Elias, 1987).

The staggered corneocyte arrangement in a lipid matrix is suggested to provide a highly tortuous lipodal diffusion pathway rendering the membrane 1000 times less permeable to water relative to other biological membranes. Due to the continuous phase, the intercellular lipid layer is considered the most important transdermal absorption pathway for small substances (Naik *et al.*, 2000). The stratum corneum by its composition and structure is considered to act as the main barrier for the exchange of substances between the body and the environment. Moreover, this anatomical barrier is accompanied by the intracutaneous metabolism, a high drainage rate due to blood and lymph capillary present in the dermis and peripheral immune system (Cevc, 2004).

10. Nanofibers

Nanofiber is characterized by long spaghetti-like masses of thin polymeric mixtures. It has been widely studied, prepared and used in various dimensions of sciences including regenerative medicine (tissue engineering scaffolds, would dressing and vascular grafts) as well as in controlled, local delivery and release of small molecule drugs and biological agents (e.g. proteins and nucleic acids) (Gupta *et al.*, 2009; Kim *et al.*, 2004; Lee *et al.*, 2008). For biomedical application, nanofibers have been prepared with synthetic or naturally derived polymers by one of three established techniques, including phase separation, self-assembly and electrospinning.

Nanofibers have been considered as long drug delivery vehicles owing to large surface area and controlled degradation. This nanomaterials have been loaded with drugs by one of three different incorporation strategies; (i) polymer-drug blends; (ii) excapsulation of water soluble emulsions; and reservoirs inside core-shell structures. Drugs have been incorporated into polyblend nanofibers by premixing the polymer

solution with the therapeutic before electrospining. The example of this polyblend nanofibers are tetracycline hydrochloride (in polyethylene-co-vinyl acetate and PLA) (Kenawy el *et al.*, 2002) and cefoxitin sodium (in PLG/PEG-b-PLA/PLA) (Kim *et al.*, 2004).