CHAPTER V
DISCUSSION AND CONCLUSION

1. Protective Effect Against Parkinson's Disease of Conventional Quercetin Administration

The present study clearly demonstrated that quercetin, a substance possessing antioxidant effect could enhance spatial working memory in Parkinson's disease (PD) rat model. In approximately 40% of PD patient is complicated by cognitive impairment (Papapetropoulos *et al.*, 2004; Williams-Gray *et al.*, 2007). Numerous studies have demonstrated that 6-OHDA is a suitable PD model to mimic the nigrostriatal damage in rodents (Blandini *et al.*, 2007; Blandini *et al.*, 2000), resulting in motor (Chaturvedi *et al.*, 2006) and cognitive deficit (De Leonibus *et al.*, 2007; Florio *et al.*, 1999; Hefco *et al.*, 2003; Hernadi *et al.*, 2000; Mura and Feldon, 2003; Tadaiesky *et al.*, 2008).

The present results demonstrated that 6-OHDA produced the spatial memory impairment in Morris water maze test and quercetin especially at dose of 300 mg/kg BW significantly produced the improvement of cognition which was in agreement with pervious studies reported that quercetin could reverse cognitive impairment induced by D-galactose and reserpine administration (Lu *et al.*, 2006; Naidu *et al.*, 2004).

It has been suggested that the ability of learning and cognitive performance was related to the density of neuron in the hippocampus (Rapp and Gallagher, 1996; Shiryaeva *et al.*, 2008). Thus, the density of survival neuron in hippocampus was determined. Quercetin particularly at high dose produced the significant improvement of neuron density in hippocampus. The precise underlying mechanism of the neurodegeneration in hippocampus induced by 6-OHDA is unclearly known. It was reported that there was a connection of mesolimbic pathway which connects between substantia nigra and the limbic system including hippocampus (Swanson, 1982), thus the damage occurring via this linkage was focused.

It was also revealed that hippocampus plays a crucial role on spatial memory (Good, 2002; Winnicka and Wisniewski, 2000). Previous studies have demonstrated that few neurotransmitter systems have been implicated in cognitive impairment in PD particularly cholinergic system (Burn and McKeith, 2003; Drachman and Leavitt, 1974; Korczyn, 2001; Perry *et al.*, 1985). It was also reported that the reduction of acetylcholine levels was related to the memory impairment (Watanabe *et al.*, 2009). Therefore, the current study was set up to evaluate the activity of acetylcholinesterase (AChE), an indirect indicator for the function of cholinergic system in hippocampus. The results showed that quercetin significantly decreased the activity of AChE. Recently, it was reported that flavonoids exhibited acetylcholinesterase inhibition effect (Jung and Park, 2007). This suggested that quercetin might exert its cognitive enhancing effect partly via the improvement of cholinergic system.

It is believed that oxidative stress plays an important role during pathogenesis of PD and, thus, MDA levels and the activity of SOD, GPX and CAT were also determined. Quercetin at all dosage range used in this study could significantly attenuate the increase in lipid peroxidation. The improvement of the activities of SOD and GPx were observed but there was no change on CAT. Therefore, it was possible that the improvement of neurodegeneration in hippocampus might not occur via the increase activity of CAT.

Rotational behavior has been recognized as a valid tool that sensitive to the nigrostriatal damage in rats (Ahmad *et al.*, 2005). However, this test is usually performed after the challenging with apomorphine, non selective dopamine agonist after being exposed to apomorphine, the rats usually developed the bias turning on the contralateral side of the lesioned side (Ungerstedt and Arbuthnott, 1970). The present data showed that following the 6-OHDA infusion and challenging with apomorphine, rats showed rotational behavior and quercetin significantly attenuated this motor change. In addition to drug induced neurobehavior change, spontaneous behavior in rats with lesion of the nigrostriatal pathway was also determined by using elevated body swing test, a simple tool perform by holding rats tail and let the rat swing its head to right and left sides (Borlongan and Sanberg, 1995). The author suggested that the possible explanation of this test might have the same mechanism to dopamine mediated rotational behavior.

Dopamine depletion in both PD brains and following nigrostriatal damage induced by 6-OHDA injection were observed. Since tyrosine hydroxylase is a rate-limiting enzyme of dopamine biosynthesis, therefore, the density of tyrosine hydroxylase positive neuron in substantia nigra was investigated by using immunohistochemistry technique. The significant destruction of dopaminergic neuron following 6-OHDA injection was observed and quercetin at dose of 300 mg/kg BW could significantly mitigate this neuronal damage. In addition to tyrosine immunochemistry, the density of nissl stained neuron in substantia nigra and striatum were also determined. The results showed that there was a decrease of neuron density in a mentioned area after 6-OHDA injection, and quercetin could significantly reverse this impairment. These results were in agreement with previous studies reported that the neurodegeneration induced by 6-OHDA was attenuated by the supplementation of dietary antioxidants (Riederer *et al.*, 1989; Roghani and Behzadi, 2001; Soto-Otero *et al.*, 2000; Zafar *et al.*, 2003).

Oxidative stress has been proposed to implicate in the pathogenesis of dopaminergic neurodegeneration in PD (Jenner, 1998; Jenner and Olanow, 1998). It has been suggested that brain lipid membranes is high vulnerable to lipid peroxidation which in turn leading to neurodegenerative disease (Balazs and Leon, 1994). Therefore, striatum was selected to determine MDA level and the activity of scavenger enzymes. The significant improvement of MDA and SOD, GPx and CAT were observed in rats which were given quercetin especially at dose of 300 mg/kg BW. In addition to oxidative stress, nitrosative stress marker such as peroxynitrie was detected following 6-OHDA injection (Henze *et al.*, 2005). This indicates that the determination of nitrogen species would provide extensive information in this PD model. Taken all data together, the current study showed that quercetin exerted the neuroprotective effect against Parkinson like symptoms. It was found that quercetin has the potential to mitigate cognitive function and motor disorders in animal model of PD induced by 6-OHDA. Its possible underlying mechanism might act partly via the improvement of oxidative stress induced by 6-OHDA.

A natural flavonoid quercetin is well known as an antioxidant which is abundant in daily consumed fruits and vegetables (Ha *et al.*, 2003; Shutenko *et al.*, 1999; Takizawa *et al.*, 2003). Quercetin was absorbed mostly in metabolites forms in

intestinal epithelial cells (glucuronides, sulfoglucuronides, sulfates and isorhamnetin) following intragastric administration in rats (Justino *et al.*, 2004). It was also found that conjugated quercetin (quercetin-3-*O*-β-D-glucuronide and quercetin-4'-*O*-β-D-glucuronide) showed the scavenging properties in copper ion-induced oxidation of human plasma LDL (Moon *et al.*, 2003). In addition, Morand and co-workers have suggested that the healthy benefits from quercetin appeared to occur due to its metabolites (Morand *et al.*, 1998). This indicates that the active substance exerting the beneficial effect should be quercetin metabolites. It has been demonstrated that quercetin sulfates and quercetin glucuronides are the main metabolites in rats after quercetin administration (Yang, *et al*., 2005).

Nevertheless, the oral bioavailability of quercetin was only approximate 20% (Hollman *et al.*, 1995; Hollman *et al.*, 1997; Ueno *et al.*, 1983) which might not enough to exert the effect on measured parameters in this experiment. However, Scalbert have suggested that quercetin do not necessarily need to be absorbed to exert an effect because the reduction of oxidative damage to DNA in cecal mucosal cells was observed after intake high concentration of tea and wine (Scalbert *et al.*, 2002). This suggested some flavonoids could exert a positive effect regardless of their poor absorption. However, since the brain is a target structure of quercetin in this study, the blood brain barrier is focused.

Blood brain barrier (BBB) is formed at the level of the endothelial cells of the cerebral capillaries and essentially comprises the major interface between the blood and the brain. Brain blood vessel endothelial cells are sealed by tight junctions, which inhibit any significant paracellular transport (Brightman and Reese, 1969; Kniesel and Wolburg, 2000; Rubin and Staddon, 1999). Specific transporters exist at the BBB permit nutrients to enter the brain and toxicants/waste products to exist (Tamai and Tsuji, 2000). The ability of substance transcytosis is depends on lipid solubility, molecular weight and charge (Habgood *et al.*, 2000). It was reported that quercetin can pass BBB (Youdim *et al.*, 2004). Thus, the active substance that exert neuroprotective effect should be quercetin or it metabolites but the further study about the precise substance that pass through the BBB is required.

The current results demonstrated the neuroprotection of quercetin against PD which was not in agreement with previous study (Kaariainen *et al.*, 2008). It was

reported that the results of 6-OHDA on the behavioral and biochemical parameters could be different depending on the site and dose of 6-OHDA administration (Schwarting and Huston, 1996). Thus, the discrepancy between our results and the previous one might be due to both parameters mentioned above because both application doses and administration route of quercetin of our study also differed from that used in the previous study.

Unfortunately the current results failed to show the dose dependent manner. The possible explanation might be related to the rapid metabolism of quercetin via the first-pass effect (Graf *et al.*, 2006) and may due to the non-simple relationship between the quercetin concentration itself and the observed parameter because quercetin might exert its influence on the observed parameters directly.

2. Protective Effect Against Parkinson's Disease of Transdermal Zein Based Quercetin Nanofiber Patch

In the recent years, transdermal drug administration (TDD) has obtained much concentration due to its advantages; decreasing the irritation of the gastrointestinal tract, avoid partial first-pass inactivation by the liver, painless and user-friendly. However, a use of TDD is still limited due to the barrier of skin particularly the stratum corneum, an outermost layer which acts as rate-limiting for transdermal transport (Badkar *et al.*, 2007).

Quercetin has the potential to exert neuroprotection in neurodegenerative disease including PD but the dosage of oral administration is relative high due to its poor absorption and rapid metabolism. Therefore, the development of zein-based biodegradable polymer loaded with quercetin is a novel strategy to administer quercetin. In the present study, transdermal zein-based biodegradable loaded with quercetin could enhance learning and memory in PD rat model and could attenuate the impairment of motor changes. The spatial working memory in rats were determined by assessing escape latency and retention time in the Morris water maze test, the results showed that quercetin nanofiber significantly improved spatial working memory. In addition, quercetin nanofiber could also improve the motor changes following the lesion in elevated body swing test and rotational behavior.

The effect of quercetin nanofiber on neurodegeneration was also investigated. In hippocampus, the quercetin nanofiber significantly attenuated neurodegeneration in all subregions of hippocampus. In addition, quercetin nanofiber also improved the neuron density in substantia nigra and striatum.

The neurodegeneration of dopaminergic neuron in the substantia nigra has been recognized as a pathological hallmark of PD. Therefore, the present study assessed the density of tyrosine hydroxylase positive neuron in this brain area. The results revealed that quercetin nanofiber significantly improved the neurodegeneration of dopaminergic neuron in substantia nigra.

During the recent years, oxidative stress has been implicated to involve with neurodegeneration (Coyle and Puttfarcken, 1993; Halliwell, 1992; Jenner *et al.*, 1992; Yoshikawa, 1993). Therefore, this study also evaluated the effect of quercetin nanofiber on the alteration of free radical homeostasis by determining the level of MDA, activity of superoxide dismutase, glutathione peroxidase and catalase in both striatum and hippocampus. The results showed that quercetin nanofiber significant improve the oxidative damage markers by decrease the level of MDA but increase activity of SOD, GSH-px and CAT. Oxidative stress is particularly detrimental to the brain where enriched with peroxidizable polyunsaturated fatty acid and has low level of catalase activity and only moderate amount of the endogenous antioxidant enzymes, superoxide dismutase and glutathione peroxidase. Therefore, the brain appears to have low capability of defensive system against oxidative stress resulting in the susceptibility to oxidative stress (Sohal, 2002). Quercetin significantly attenuated oxidative stress induced neurodegeneration (Heo and Lee, 2000) by regulating the reactive oxygen species by increased activity of GPx (Sun *et al*., 2006), SOD but decreased level of MDA (Lu *et al.*, 2006) in aging model induced by D-galactose. Previous studies have suggested that the antioxidant effect of quercetin was demonstrated to involve with the metal chelation, the activation of GPx. This suggested that the improvement of oxidative status of quercetin is appears to relate with its antioxidant effect.

The mechanism of quercetin loaded zein based polymer to produce the positive effect was believed to be similarly to quercetin conventional administration. Although, both conventional administration of quercetin and the transdermal

application of quercetin-loaded zein-based polymer can provide the same beneficial effect. The effective dose via transdermal route application of quercetin loaded zein-based polymer is approximately 100 times lower than that of the oral administration. While the effective dose of oral administration of quercetin is 300 mg/kg BW, the calculated dose of quercetin delivered by transdermal application of quercetin loaded zein based polymer is only 3 mg/kg/day (quercetin nanofiber at concentration 5%, 10% and 15% were equal to 1, 2 and 3 mg/day).

Based on the previous information on physicochemical of substance possessing the ability to deliver through the skin; molecular weight should be less than 500 Da, the dose of substance should be less than 10 mg/day and exhibit solubility (Naik *et al.*, 2000). Quercetin could pass through the skin barrier because its molecular weight is only 302 Da and its structure is the semi-soluble. In addition, the dose of quercetin loaded in nanofiber in this study was only 3 mg/day. Therefore, it appears to fit the criteria. Quercetin should be able to penetrate through the skin via passive diffusion. Tojo and colleague have reported that drugs permeate through the viable skin is rapidly absorbed by microcirculation (Tojo *et al.*, 1987). After successfully penetrated through the skin and diffused to the blood stream underneath the skin, quercetin or its metabolites would diffuse into the systemic circulation which the active substance must face to the BBB which is known to be a very restricted structure and with a selective, hydrophobic barrier which allow the small and lipid soluble molecules to pass though (Oldendorf, 1975).

It was reported that the biotransformation of quercetin may affect its function and activity because its metabolites are generally more hydrophilic and have a negative charge at physiological pH (Williams and Elias, 1987; Williamson *et al.*, 2000). This is indicating that the penetration pass through the BBB of quercetin or its metabolites may not be the main pathway. Therefore, another mechanism of quercetin to exert the effect should be focused.

The precise active substance that produced these all positive results of the current study is unknown because it is less information about the diffusion of quercetin trough the BBB is available. Nevertheless, it was suggested that the neuroprotection produced by flavonoids may act directly on cell signaling (Moon *et al.*, 2003; Schroeter *et al.*, 2002). In addition, it has been demonstrated that

oxidative stress has a diverse effect on signaling pathways in cells, such as MAP kinase pathway (mitogenic ERK1/2, the stress-activated JNK and p38 cascades) (Blanc *et al.*, 2003; Torres and Forman, 2003) which play a key role in the regulation of various functions include neuronal apoptosis (Kim and Iwao, 2003; Xia *et al.*, 1995). JNK, a pro-apoptotic signaling protein, has been strongly linked to transcription-dependent apoptotic signaling (Mielke and Herdegen, 2000; Yuan and Yankner, 2000). This signaling protein is also activated by dopamine in oxidative stress condition (Luo *et al.*, 1998). It was demonstrated that quercetin exert the anti-apoptotic effect by suppression JNK and ERK (Ishikawa and Kitamura, 2000). In addition, the characteristic of neuronal death induced by 6-OHDA in substantia nigra was noted to be apoptotic manner (Marti *et al.*, 1997). Taken all pieces of information together suggested that quercetin might exert its neuroprotection by inhibition the apoptotic signaling molecule. However, the further study about this idea is required.

3. Conclusion

Quercetin, a natural flavonol commonly found in daily consumed fruits and vegetables provides beneficial effect on cognitive enhancing effect, motor improvement and neuroprotection in PD model induced by 6-OHDA. These all effects may occur partly via the increase in the density of neurons (hippocampus, substantia nigra and striatum), and the density of tyrosine hydroxylase positive neuron in substantia nigra due to the improvement of antioxidant system function of all scavenger enzymes (SOD, GPx and CAT) which in turn decrease the level of MDA. In addition, the alteration of cholinergic system as observed by a decrease in AChE activity was also involved.

The development quercetin in zein-based biodegradable nanofiber was useful because this form provided the effectiveness with low dose leading to low risk of toxicity induced by the high amount of administered substance. Therefore, quercetin-loaded zein based nanofiber is the potential candidate for neuroprotective against Parkinson's disease. However, the further study especially toxicity is required before human application.