INTRODUCTION

Most insights into the pathogenesis of Parkinson’s disease (PD) come from investigations done in experimental models of Parkinson’s disease. Most of the animal models used in PD produce the neurodegenerative disorder using neurotoxins. These neurotoxins are capable of causing oxidative toxicity and death of the dopaminergic neurons (Cannon and Greenamyre, 2010). Development of PD in various animal models has been achieved by administration of different neurotoxins which are capable of destroying or disrupting catecholaminergic system selectively including the use of 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone and paraquat. 6-OHDA, remains the most popular animal model of PD for the assessment of new therapeutic options (Cicchetti et al., 2009; Uversky, 2004).

There is a need for alternate therapeutic agents that effectively prevent the progression of neurodegenerative processes such as ‘neuroprotective’ or ‘disease-modifying’ treatments aiming at slowing down the disease progression. Quercetin (QN), is a bioflavonoid found in abundance in fruits and vegetables, especially citrus fruits, apples, onions, parsley, tea, red wine and also olive oil, grapes, dark cherries and dark berries. Quercetin is regarded as one of the most potent antioxidants among all the flavonoids, which is capable of scavenging reactive oxygen species and binding transition metal ions (e.g., iron and copper); hence it protects the body from oxidative damage (Loke et al., 2008; Miean and Mohamed, 2001).

Desferrioxamine (also known as desferrioxamine, DFO, or desferal) is a specific chelating agent derived from ferric oxide. It has been used clinically to treat iron toxicity, including acute iron poisoning and iron-overload.
anemia, such as thalassemia major, as well as aluminium poisoning associated with chronic renal dialysis. The role of desferrioxamine as an antioxidant and free-radical scavenger in non-iron intoxications has been revealed in vitro study that iron-driven hydroxyl radical formation pathway in γ-irradiated cells were blocked by desferrioxamine at different nontoxic concentrations (Guelman et al., 2004). The neuroprotective effect of desferrioxamine has been proposed in various cerebral ischemia models by several authors (Nakamura et al., 2004; Okauchi et al., 2009). The present study is aimed to evaluate the neuroprotective effect of combined administration of quercetin and desferrioxamine in 6-OHDA – induced neurotoxicity in adult male rats.

MATERIALS AND METHODS

Chemicals

Protein carbonyl (PC), total glutathione (GSH) superoxide dismutase (SOD) assay kits were purchased from Cayman Chemicals (Cayman Chemicals and Pierce Biotechnology, Rockford, IL, USA). Dopamine assay kit was obtained from GenWay Biotech (San Diego, CA, USA). 6-hydroxydopamine (6-OHDA), quercetin (QN) and desferrioxamine (DFO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Experimental animals

Adult male, Sprague-Dawley rats, with body weight ranging between 200-250 g were purchased from animal holding facility, University Kebangsaan Malaysia (UKM), Kuala Lumpur, Malaysia. The rats were maintained in an air-conditioned room (25 ± 2°C), at 12 hr: 12 hr light-dark cycle and fed with standard diet and water ad libitum. All experimental procedures were in accordance with the International Ethical Guidelines for Animal Experimentation, and the study protocol was approved by the Institute Research and Ethical Committee.

Experimental design

The rats were weighed and randomly assigned into six groups (6 rats per group); group I – control (without any treatment), group II – sham lesion group, group III – 6-OHDA group (300 μg of 6-OHDA, injected intracisternally), group IV – 6-OHDA with QN (50 mg/kg body weight, orally) for 14 days, group V – 6-OHDA with DFO (50 mg/kg body weight, intraperitoneally) for 14 days and group VI – 6-OHDA with QN and DFO, concurrently for 14 days.

6-OHDA injection

Rat was anesthetized under light ether anesthesia. All the procedures were carried out under aseptic condition. Rat was fixed to stereotaxic board and 10 μl of 6-OHDA solution containing 300 μg of 6-OHDA was then slowly administrated intracisternally. Sham lesion group of rats received 10 μl of normal saline through intracisternal injections. Animals were allowed 48 hr of recovery before the initiation of treatment. During the recovery period, the rats were observed for the presence of seizure, hypokinesia, rigidity, tremor, purposeless chewing, piloerection and also the changes in body weight.

All the experimental groups were maintained for 14 days before they were sacrificed. Changes in body weight, food intake, and behavioral study parameter using paw retraction test were evaluated. Total food intake was measured as the differences between the amount of food and water given and the amount left in the cages on the subsequent day. Behavioral changes were accessed using paw retraction test.

Behavioral test

The behavioral abnormalities induced by 6-OHDA was accessed two days after the procedure using ‘paw retraction test’ which is a test for akinesia as previously described (Ellenbroek et al., 1987). The forelimb retraction time (FRT) was defined as the time it took for the rat to retract the forelimb. Similarly, the hind limb retraction time (HRT) was defined as the time it took for the rat to retract the hind limb. Minimum retraction time was set to one second and maximum time was thirty seconds. Two trials were performed each time the test was carried the individual score was calculated as the mean of the two trials for each test.

Biochemical and histological study

At the end of the treatment period, the final body weight, and amount of food intake were recorded. Rats were sacrificed with an overdose of sodium pentobarbital (70 mg/kg body weight), and the dissection of the brain was done immediately. Right half of the brain was used for qualitative study of the histological changes which was fixed immediately in 10% formalin and left half of the brain was used for biochemical analysis. The corpus striatum was carefully removed and then homogenized using the tissue grinder (Duell®, all glass) in ice to ensure the protein in the samples were not denatured by heat. The homogenates were subsequently centrifuged, and the supernatant was collected and stored at -80°C.

From the homogenate samples, protein carbonyl (PCC), superoxide dismutase (SOD), glutathione...
(GSH) levels were assayed using ELISA kits (Cayman Chemicals and Pierce Biotechnology). Dopamine levels of the samples were estimated by ELISA kits from GenWay Biotech.

**Protein carbonyl content (PCC) assay**

In the protein carbonyl content assay kit (Cayman Chemicals), protein samples are derivatized by making use of the reaction between 2,4-dinitrophenylhydrazine (DNPH) and protein carbonyls. Formation of a Schiff base produces the corresponding hydrazone which was analyzed spectrophotometrically at 360-385 nm.

**Superoxide dismutase (SOD) assay**

This assay kit (Cayman Chemicals) utilizes a tetrazolium salt for the detection of superoxide radicals (O$_2^-$) generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme necessary to exhibit 50% dismutation of the superoxide radical. Oxidation rate of tetrazolium salt to formazan dye by O$_2^-$ is inversely proportional to the endogenous activity of SOD. The formazan dye stains the wells and its staining intensity was detected by absorbance spectrophotometry at 450 nm.

**Glutathione (GSH) assay**

Cayman chemicals glutathione enzyme-linked immunosorbent assay utilizes enzymatic recycling method, using glutathione reductase, for the quantification of glutathione. The sulphydryl group of GSH reacts with 5,5'-dithio-bis-2-nitrobenzoic acid and produces a yellow coloured 5-thio-2-nitrobenzoic acid (TNB). The rate of TNB production is proportional to the recycling reaction, which in turn directly indicates the concentration of glutathione in the sample. 5-thiol, 2-nitrobenzoic acid absorbance was measured at 405 nm, to estimate the glutathione in the sample. The glutathione concentration in the sample was determined by end point method using the standard curve.

**Dopamine assay**

Solid phase enzyme-linked immunosorbent dopamine assay based on the sandwich principle. The wells were coated with a goat anti-rabbit antibody. The added liquid antibody, directed towards an epitope of an antigen molecule binds to the plate within the incubation time. The antigen of the sample was incubated in the coated well with enzyme conjugated second antibody (E-Ab), directed towards a different region of the antigen molecule. After the substrate reaction the intensity of the developed color was proportional to the amount of the antigen. The color intensity was detected using absorbance spectrometry at 405 nm (Reference-wavelength: 620-650 nm). Results of samples were determined directly using the standard curve (GenWay Biotech).

For histology, the brain samples obtained were processed into paraffin blocks using a tissue processor. Sectioning was done using a rotary microtome (LEICA RM2235) at a thickness of 5 μm and was stained. The stained sections containing the striatal area were studied using Nikon Brightfield Compound Microscope, YS100 (attached with Nikon cameral) and software NIL Element BR 3.00. Neuronal count was then performed in the regions of the striatum as mentioned in methods within a measured rectangular area of 300 x 300 μm$^2$ (Laursen and Diemer, 1979).

**Statistical analysis**

All data were presented as means ± S.D. Statistical comparisons between different treatments were performed using one-way analysis of variance (ANOVA) with Bonferroni’s multiple comparison at p < 0.05.

**RESULTS**

**Body weight and food intake**

A significant decrease (p < 0.05) in body weight was observed in all 6-OHDA treated rats after receiving intracisternal administration of 6-OHDA compared to control group. When the mean of body weight was analysed, a significant difference in body weight changes between control and all the 6-OHDA treated groups was found (p < 0.05). There was a significant recovery (p < 0.05) in body weight in combined treatment groups after 14 days, which was significantly higher from 6-OHDA alone and 6-OHDA with QN groups. As shown in Fig. 1, 6-OHDA alone group showed a significant decrease in food intake after intracisternal injection of 6-OHDA (p < 0.05). QN treatment resulted in a small increase in the amount of food intake, and similar finding was observed in rats receiving DFO alone for 14 days. Combined treatment significantly increased the food intake (p < 0.05) more than the QN or DFO alone group. All the rats with 6-OHDA administration showed a significant decrease in food intake (Figs. 1 and 2).

**Paw retraction time**

There was a significant increase in latency of FRT and HRT between control (p < 0.05) and 6-OHDA alone treated group. Following treatment with quercetin, desferrioxamine, and combined treatment of both, a significant improvement (p < 0.05) in FRT and HRT was observed.
Combined treatment revealed more significant improvement on FRT and HRT (p < 0.05) after 14-day treatment (Fig. 3).

Biochemical assays
Following 6-OHDA administration, a significant decrease in the striatal dopamine level by 25% (p < 0.05) was seen. Following treatment with quercetin, desferrioxamine or combined treatment of both, a significant increase (p < 0.05) in striatal dopamine level was observed. Following the desferrioxamine treatment, the dopamine level was increased by 12% (p < 0.05). Quercetin seemed to have more protective effect against 6-OHDA induced DA fall compared to desferrioxamine. On the other hands, greatest increase in dopamine level was observed in combined treatment with both quercetin and desferrioxamine by 25% (p < 0.05) compared to the 6-OHDA alone group. Moreover, a 12% more increase in DA level compared to desferrioxamine treatment, and a 4% more increase in DA level compared to quercetin treatment was observed after combined treatment of both for 14 days (Fig. 4).

As shown in Fig. 5, 6-OHDA alone treated group showed a significant elevation of protein carbonyl content compared to control group (p < 0.05). Following
treatment with quercetin, the protein carbonyl content was reduced significantly by 40% (p < 0.01). On the other hand, combined treatment with quercetin and desferrioxamine more significantly reduced the protein carbonyl content by about 60% (p < 0.05). Furthermore, when compared to quercetin treatment, combined treatment resulted in more reduction in protein carbonyl content (Fig. 5).

The total GSH level in the brain was found to be significantly decreased after a single dose of intracisternal administration of 6-OHDA when compared to control group (p < 0.05). Rats receiving quercetin treatment showed a significant elevation of the striatal GSH level (p < 0.05) compared to the 6-OHDA alone group. Combined treatment with quercetin and desferrioxamine resulted in a more significant increase in GSH level in the striatum (p < 0.05). Treatment with quercetin showed a greater increase in GSH level compared to that of desferrioxamine (p < 0.05) (Fig. 6).

There was a significant decrease (p < 0.05) in striatal super oxide dismutase level in the 6-OHDA alone group compared to control group. Treatment with QN and DFO significantly increased (p < 0.05) SOD level than 6-OHDA alone. There was a more significant increase (p < 0.05) in SOD in the striatum in the combined group than QN alone and DFO alone group. Compared to the DFO alone, QN alone has more significant increase (p < 0.05) in SOD level (Fig. 7).

Histopathological changes
There was a significant decrease in neuronal density of the striatum (caudate-putamen complex) after administration of 6-OHDA (p < 0.05). Following treatment with quercetin, striatal neuron’s density increased significantly (p < 0.05) compared to the 6-OHDA alone group. Combined treatment with quercetin and desferrioxamine significantly increased the neuronal density of the striatal neurons by about 45% (p < 0.05) compared to the 6-OHDA alone treated group. Furthermore, when compared with quercetin and desferrioxamine treated groups, more significant improvement in neuronal density was observed after combined treatment (Fig. 8). Control group revealed healthy, normal-looking neurons (medium spiny projection neuron). Intact of the integrity of the cell membrane was observed in majority and the cell body of the neurons contained a large nucleus with
crisp nuclear membrane, dispersed chromatin, prominent nucleolus and peri-nuclear Nissl granules. There was no evidence of apoptotic changes and very minimal pyknosis of the neurons was found. Neurons appeared in adequate numbers and distributed evenly in the area of caudate-putamen complex (Fig. 8A). There was no evidence of pathological changes in the histological section in sham lesion group (Fig. 8B). The 6-OHDA alone treated group showed a marked reduction in numbers of normal looking neurons in striatum compared to control group. Increased pyknosis of the neurons and loss of integrity of cell membrane was found. Loss of peri-nuclear Nissl granules and loss of prominent nucleolus was also present (Fig. 8C). The quercetin treated group with 6-OHDA administration showed a significant increase in the number of normal-looking neurons. Pyknosis was present in a lesser extent compared to the 6-OHDA alone treated group (Fig. 8D). Increased number of normal-looking neurons compared to the 6-OHDA alone treated group was seen after 14-day treatment with desferrioxamine (Fig. 8E). The combined treatment group showed a more significant increase in numbers of normal-looking neurons compared to quercetin alone or desferrioxamine alone treated group. Pyknosis was present in lesser extent compared to the striatal region of rats, which received only quercetin or desferrioxamine treatment (Fig. 8F).
Neuroprotective effects of quercetin and desferrioxamine

**DISCUSSION**

In the present work, a significant reduction in food intake in all the 6-OHDA treated groups was recorded when compared to control group and sham lesion group, which is in consistent with the previous findings (Blum et al., 2001; Deumens et al., 2002; Truong et al., 2006). A significant weight loss was observed in all the 6-OHDA treated rats two days after the procedure which gradually increased in the next few days, supporting the earlier observation by Breese and Traylor (1971). The above findings could be due largely to the toxic effect of the 6-OHDA on the central nervous system (CNS) which in turn causes remarkable motor impairment as evidenced by increase latency in both FRT and HRT in paw retraction test after 6-OHDA administration. The resultant impairment in food and water intake apparently contributed to the weight loss observed after 6-OHDA treatment. Present study showed that rat’s receiving quercetin, desferrioxamine or both, returned to normal-weight gain more rapidly compared to rats that received only 6-OHDA.

With increased loss of nigrostriatal functions by injection of intracisternal 6-OHDA, significant motor impairment was seen in rats as manifested by increased latency in paw retraction test. The test has been shown to be able to detect small bilateral lesions produced changes in behavioral parameters that are specific to caudate-putamen complex (He et al., 2000). In the present study, a significant increased duration in both forelimb and hind limb retraction time was observed after 6-OHDA treatment. Following treatment with antioxidant quercetin and desferrioxamine, the rats could recover in their motor function with combined treatment showing a significant recovery in paw retraction test.

Intracisternal or intraventricular injection of 6-OHDA has been shown to induce prolonged depletion of brain catecholamine presumably due to its selective destruction of CNS without interfering with the peripheral stores (Sing et al., 2003; Storch et al., 2001). These studies are comparable to the findings of the present study that the administration of 6-OHDA suppressed the striatal DA level drastically after 14 days. The result obtained could be due, to the acute toxic effect of 6-OHDA on dopaminergic neurons, which significantly affect the normal physiological activity of the neurons. Upon administrated into CNS, 6-OHDA is taken up by dopaminergic terminals and retrogradely transported into the cell body where it inhibits mitochondrial complex, I activity leading to apoptotic cell death (Cannon and Greenamyre, 2010; Uversky, 2004). Quinones are formed during auto-oxidation of 6-OHDA inside the neurons which inactivate some of the biological macromolecules by reacting with their nucleophilic groups, and also causes free radical generation (Deumens et al., 2002). Following treatment with an antioxidant and iron chelator, a lesser degree of brain dopamine loss was detected in the present study. Elevation of dopamine level after quercetin treatment was likely to be the result of improved neuronal survivability and also the possible COMT or MAO inhibitory activities of quercetin, which may reduce the dopamine breakdown, hence preserve the neuronal dopamine level (Ben-Shachar et al., 1991). Desferrioxamine also seemed to increase the dopamine level probably through its iron chelating action reducing the accumulation of redox-active iron, which is toxic to the dopaminergic neurons (Yuan et al., 2008).

The result of this study provided evidence that the antioxidant, quercetin, is capable of decreasing the oxidative load on neuronal cells in the striatum as evidenced by decrease in protein carbonyl content and increase in total GSH and SOD levels (David et al., 2008). Desferrioxamine also provided a significant protection against 6-OHDA induced fall in striatal GSH, SOD and increase protein carbonyl content. These antioxidant enzymes are involved in the defense system against free radical mediated tissue or cellular damage (Reiter, 1998). The observed decrease in striatal antioxidants and increase in protein carbonyl contents confirm the 6-OHDA induced oxidative stress and quercetin, and desferrioxamine successfully reduced the oxidative insult in the present study.

The neurotoxicity of 6-OHDA has been partially associated with its ability to release ferritin-bound iron hence increase its concentration at the site where it initiates the neurodegenerative process (Andringa et al., 2000). Mitochondrial disruption and oxidative stress are the two main causes for the neuronal death in 6-OHDA neurotoxicity. The finding that administrations of DFO significantly reduced the PCC, in the brain region with the increase in dopamine, GSH and SOD activity, strongly suggests that this agent was capable of crossing BBB at a considerable level and reach the brain region, in agreement with the earlier reports on the beneficial neuroprotective effect of DFO in reducing excessive iron content in brain region (Lan and Jiang, 1997). Several studies have demonstrated the role of DFO in promoting neuronal protection. But the detail mechanism of how DFO treatment influences protection against neurotoxicity is not known. Iron is an important contributor to Parkinson’s pathology by inducing oxidative stress and the aggregation of alpha synuclein and other proteins. Iron chelator DFO may improve neuronal survival by chelating excessive iron and reducing oxidative stress in the neurons (Glinka et al., 1998).

In this study, combined pretreatment with QN and
DFO, maintained the neuronal number and reduced oxidative stress in the striatum. Combined treatment with QN and DFO reduced the neuronal loss by their inhibitory effect on lipid peroxidation and increasing antioxidant enzyme levels in the striatum. The study has demonstrated that combined treatment was better than single treatment. A combination of antioxidant and iron chelator may be useful for the treatment of 6-OHDA neurotoxicity or in Parkinson’s disease, which is due to oxidative stress and iron accumulation (Grünblatt et al., 1999).

This neurotoxicity study with 6-OHDA had some limitations. At higher concentrations, 6-OHDA administered intracisternally might have caused non-specific damage of the catecholaminergic neurons. Therefore, some of the rapid behavioural changes observed in the animals could be due to loss of other catecholaminergic neurons in the brain. Normally, 6-OHDA lesions are achieved by administration of the drug directly into the medial forebrain bundle. However, this will produce complete striatal dopaminergic neuronal loss very quickly. Present study looked into the neuronal loss and dopamine level in the striatum instead of specific areas like substantia nigra.

In conclusion, intracisternal injection of 6-hydroxydopamine produced a significant striatal neurotoxicity with depletion of dopamine and antioxidant enzymes. Treatment with quercetin and desferrioxamine could reduce these changes significantly with increased antioxidant enzymes and retaining striatal neurons. Combined treatment with quercetin and desferrioxamine had a more significant effect in reducing the 6-OHDA – induced oxidative stress and neuronal damage. Hence, flavonoid quercetin and desferrioxamine may be of therapeutic value in protecting neurons against drug – induced neurotoxicity. The molecular mechanism of quercetin and desferrioxamine – induced neuroprotection is being investigated.

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