Rutin, a bioflavonoid antioxidant protects rat pheochromocytoma (PC-12) cells against 6-hydroxydopamine (6-OHDA)-induced neurotoxicity

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Abstract. Free radicals are widely known to be the major cause of human diseases such as neurodegenerative diseases, cancer, allergy and autoimmune diseases. Human cells are equipped with a powerful natural antioxidant enzyme network. However, antioxidants, particularly those originating from natural sources such as fruits and vegetables, are still considered essential. Rutin, a quercetin glycoside, has been proven to possess antioxidant potential. However, the neuroprotective effect of rutin in pheochromocytoma (PC-12) cells has not been studied extensively. Therefore, the present study was designed to establish the neuroprotective role of rutin as well as to elucidate the antioxidant mechanism of rutin in 6-hydroxydopamine (6-OHDA)-induced toxicity in PC-12 neuronal cells. PC-12 cells were pretreated with different concentrations of rutin for 4, 8 and 12 h and subsequently incubated with 6-OHDA for 24 h to induce oxidative stress. A significant cytoprotective activity was observed in rutin pretreated cells in a dose-dependent manner. Furthermore, there was marked activation of antioxidant enzymes including superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and total glutathione (GSH) in rutin pretreated cells compared to cells incubated with 6-OHDA alone. Rutin significantly reduced lipid peroxidation in 6-OHDA-induced PC-12 cells. On the basis of these observations, it was concluded that the bioflavonoid rutin inhibited 6-OHDA-induced neurotoxicity in PC-12 cells by improving antioxidant enzyme levels and inhibiting lipid peroxidation.

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Introduction

Antioxidant compounds are reducing agents that arrest the progression of chain reaction by scavenging the free radicals and protect cells from undergoing degeneration (1). Nevertheless, a powerful antioxidant enzyme network exists in all aerobic organisms that deals with reactive oxygen species (ROS), including hydroxyl radicals (OH), and superoxide anions (O_2^-). Antioxidant enzymes such as Cu/Zn superoxide dismutase (SOD), Mn-SOD, catalase, glutathione peroxidase (GPx) and glutathione (GSH) protect cells by catalyzing the perilous ROS into less hazardous compounds (2). Apart from the endogenous antioxidant system, antioxidants derived from plant sources also play a key role in maintaining the equilibrium between cellular ROS production and internal defense against oxidative stress (1,2).

Flavonoids are naturally occurring polyphenols found ubiquitously in various fruits, leaves and seeds (3). Rutin, a bioflavonoid compound, is a glycoside derivative of quercetin, a polyphenol well known for its anticancer (4), anti-inflammatory (5) anti-viral (6) and antioxidant (7) effects. The chemical structure of rutin (Fig. 1) resembles that of quercetin, with the exception that the hydrogen atom on the right side in quercetin is replaced by the disaccharide rutinose (rhamnose and glucose) molecule in rutin and is also known as quercetin-3-O-rutinoside (8). Rutin is widely found in citrus fruits and the rinds of grapes and lime, in berries, including cranberries and mulberries (9), as well as buckwheat and asparagus (8). Although many studies have proven the neuroprotective role of quercetin, attention to rutin has been lacking. Rutin has disaccharide sugar molecules as the side chain (Fig. 1). Therefore, rutin may exhibit improved antioxidant properties as well as greater bioavailability potential as compared to quercetin (10).

Although evidence suggests an association between rutin and its neuroprotective activity, the antioxidant mechanism of rutin is not well established. Thus, the objective of this study was to establish the neuroprotective role of rutin as well as to elucidate its antioxidant mechanism by specifically observing its role in altering the natural antioxidant enzyme network in 6-hydroxydopamine (6-OHDA)-induced neurotoxicity in

pheochromocytoma (PC-12) neuronal cells. PC-12 cells are commonly used in the investigation of neurotherapeutics study for Parkinson's disease (PD). PC-12 cells are known to secrete dopamine neurotransmitters and contain high amounts of dopamine transporters. In this experimental model, 6-OHDA (a hydroxylated analogue of dopamine) was used to induce neurodegeneration. 6-OHDA-induced neuronal death provides the more comparable event of PD as in human brains (11).

Materials and methods

Materials. PC-12 cells were purchased from ATCC (#CRL-1721.1 PC-12 ADH, *Rattus norvegicus*, Manassas, VA, USA). 6-OHDA, rutin, poly-L-lysine, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], and dimethyl sulphoxide (DMSO) were purchased from Sigma Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), pen-strep, horse serum, and fetal bovine serum (FBS) were purchased from Gibco (Carlsbad, CA, USA). Antioxidant enzyme kits, GPx SOD, catalase, thiobarbiturate, and GSH assay kits were purchased from Cayman Chemicals.

Cell culture. PC-12 cells were grown in a humidified incubator with 5% CO_2 at a temperature of 37°C in DMEM medium supplemented with 5% horse serum and 5% FBS and pen-strep (100 U/ml). The cells were cultured in poly-L-lysine-coated T-75 culture flasks. The cells used in the experiments were taken between passages 3 and 8, as cells become clumpy and difficult to singularize after passage 10. When the cells were 60% confluent, they were dislodged from the flask using cell scraper. Subsequently, the cells were dispersed by vigorous pipetting in and out for several times. The dispersed cells were plated on a poly-L-lysine-coated 96-well microplate at a density of $1x10^5$ cells/ml and incubated overnight in order to facilitate cell adhesion to the substrate. The cells were then cultured for 4, 8 and 12 h in the presence of rutin at 10, 50 and 100 μM. Subsequently, the pretreated cells were induced using 6-OHDA for 24 h and assayed for its antioxidant activities. Control cells were not treated with rutin or 6-OHDA, while positive control cells were treated with 6-OHDA only. Rutin was dissolved in DMSO and then diluted with complete culture media. A concentration of DMSO in the final culture media of <0.05% had no protective or damaging effects on PC-12 cells.

Preparation of 6-OHDA stock solution. 6-OHDA-HBr (1 mg) was dissolved in 2 ml of chilled 0.15% of ascorbic acid to produce up to 2 mM of stock solution. The 0.15% of chilled ascorbic acid arrested the oxidation of 6-OHDA-HBr. The tube used to dissolve 6-OHDA was covered with aluminium foil to protect the stock solution from intense light exposure. The stock solution was subsequently filtered using a 0.2 μm syringe filter and diluted to the desired concentration using complete culture media.

Determination of cell viability. The cytotoxicity effect of rutin on 6-OHDA-induced PC-12 was determined using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. MTT, a yellow tetrazole, was reduced to insoluble purple formazan in the mitochondria of viable cells. The insoluble purple formazan was dissolved using a solubilizing solvent and the colored solution was measured at 570 nm using a microplate reader. Following the incubation of PC-12 cells with different concentrations of rutin at different time points, 10 μl of MTT (5 mg/ml) was added to each well and incubated for 4 h. The supernatant was removed, and 100 μl DMSO was added to solubilize the insoluble purple formazan. The absorbance was read at 570 nm using the Opsys microplate reader (DYNEX Technologies, Chantilly, VA, USA). Data on cell viability were expressed as a percentage of the surviving control cells in the study.

Biochemical analysis. Each sample of treated and untreated PC-12 cells was collected using cold phosphate-buffered saline (PBS) by centrifugation at 4°C at the specific centrifugation speed required by each enzyme kit. The cell pellet was homogenized using cold buffer and the clear supernatant was obtained for the measurements of GSH, GPx, SOD, and catalase and malondialdehyde (MDA) activities.

GSH assay. The sulfhydryl group of GSH in the sample reacted with DTNB [5,5'-dithio-bis-2-(nitrobenzoic acid)] generating a yellow-colored TNB (5-thio-2-nitrobenzoic acid). Glutathione reductase reduced the disulphide mixture (GSH and TNB), producing an increased amount of TNB. The rate of TNB production was directly proportional to the amount of GSH present in the sample. GSH concentration was determined by measuring the absorbance of TNB at 405-414 nm using a microplate reader.

GPx assay. GPx enzyme catalyzed the reduction of hydroperoxides by using two molecules of reduced GSH to produce one molecule of reduced hydroperoxides and oxidized glutathione (GSSG). This reaction was coupled with a second reaction which recycled GSSG into its reduced state by glutathione reductase and NADPH. The oxidation of NADPH to NADP+ was followed by a decrease of absorbance at 340 nm, which was measured every 1 min for 8 min and was directly proportional to the GSH activity in the sample.

SOD assay. Total SOD activity (cytosolic and mitochondrial) in the sample was assayed using a Cayman Chemical assay kit. SODs are metalloenzymes that catalyze the dismutation of free radicals, specifically superoxide anion, to less toxic compounds including oxygen and hydrogen peroxide molecules. The superoxide anion radicals in the reaction were generated by hypoxanthine and xanthine oxidase. The dismutation reaction by SOD utilized the tetrazolium salt resulting in color change and the quantity of SODs was measured at 440 nm using a microplate reader.

Catalase assay. Catalase enzyme activity is usually estimated by catalytic activity. However, for the Cayman Chemicals enzyme kit, the catalase activity was measured using peroxidatic activity. In the peroxidatic reaction, catalase mediated the reaction between hydrogen peroxidase and low molecular alcohol, which served as electron donors for the production of water and formaldehyde molecules. The formaldehyde molecules reacted with the chromogen, 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald), resulting in purple color

change and were measured colorimetrically at 540 nm using a microplate reader.

Lipid peroxidation assay. Lipid peroxidation was determined by the thiobarbituric acid reactive (TBARS) method. The cell lysates were mixed with thiobarbituric acid (TBA) and allowed to react at a temperature of 95-100˚C. The naturally occurring product of lipid peroxidation, MDA reacted with TBA, forming the MDA-TBA adduct, an acidic condition that was measured colorimetrically at 540 nm using a microplate reader.

Statistical analysis. The experiments were repeated three times in triplicate. Results were shown as the mean ± SEM. Data were analyzed using one-way ANOVA and SPSS Inc. software (SPSS Statistics Desktop, V20.0.0). P<0.05 was considered statistically significant.

Results

Dose response of 6-OHDA toxicity. Cell viability markedly decreased following a 24-h incubation of PC-12 cells with an increasing concentration of 6-OHDA (0-200 μM). The dose response study was crucial to determine the IC_{50} value of the 6-OHDA concentration. The 6-OHDA concentration, which resulted in 50% PC-12 cell inhibition, was 100 μM (50.33±1.72) on $1x10^4$ cells/ml. The cell viability of each sample concentration was compared with the mean percentage of the untreated control and reported as the mean ± SEM (Fig. 2).

Effect of rutin in 6-OHDA-induced oxidative stress in PC-12 cells. Results of MTT assay following rutin pretreatment showed a significant increase in cell viability in a dose-dependent manner. The 100 μM of 6-OHDA treatment caused 50% cell inhibition (50.33%±1.72) on $1x10^4$ cells/ml of PC-12 cells. Thus, 100 μM of 6-OHDA was used for the subsequent experiments to assess the protective effect of rutin. The 100 μM of 6-OHDA resulted in cell viability of 56.92±2.31. The cell viability was the highest at 100 μM of rutin at all three time points. However, the optimum reading was recorded at 100 μM of rutin at 8 h of pretreatment (80.06±3.94). However, the protective ability of rutin decreased after 12 h of incubation (Fig. 3).

SOD activity following rutin pretreatment. SOD level significantly increased in all the rutin pretreated PC-12 cells. Test groups showed a statistically significant difference compared to 6-OHDA alone (P<0.01). The SOD activity showed a positive trend with increasing rutin concentration (Fig. 4).

Catalase level following rutin pretreatment. Catalase level was significantly increased in all the rutin pretreated groups and the trend was directly proportional to the rutin concentration. Almost all data showed a statistically significant value in comparison with 6-OHDA alone (Fig. 5).

GSH level following rutin pretreatment. The internal GSH in response to different concentrations of rutin yielded significant (P<0.01) positive results. The GSH level was significantly
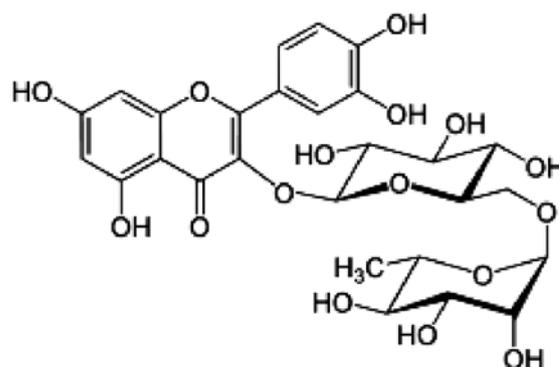

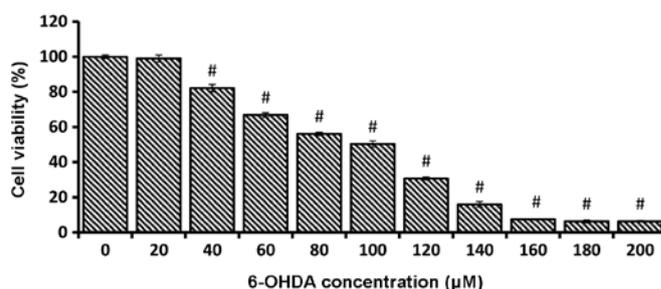Figure 1. Rutin (quercetin-rhamnose).

Figure 2. The dose-response of different concentrations of 6-hydroxydopamine (6-OHDA) (0-200 μM) used to induce neurodegeneration in pheochromocytoma (PC-12) cells for the duration of 24 h. Data are representative of mean ± SEM (n=4) of three independent experiments. #P<0.01 vs. negative control.
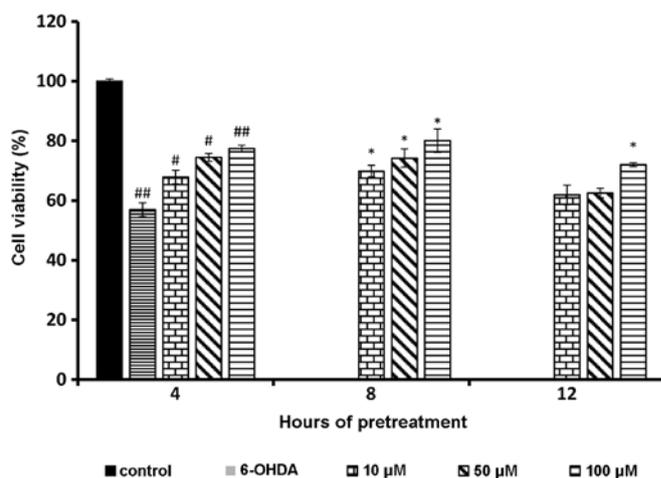

Figure 3. Cell viability by MTT assay after pretreatment with rutin for 4, 8 and 12 h and subsequently with 100 μM of 6-hydroxydopamine (6-OHDA) for 24 h. Values are presented as mean ± SEM. (n=4). *P<0.05, #P<0.01 and ##P<0.001.

elevated in the untreated sample and decreased in the sample incubated with 6-OHDA alone. Of note, the samples incubated with rutin demonstrated significantly higher amounts (P<0.01) of GSH concentrations. The GSH concentration was indirectly proportional to the rutin concentration. As the rutin concentration was increased the cells produced a reduced amount of GSH (Fig. 6).
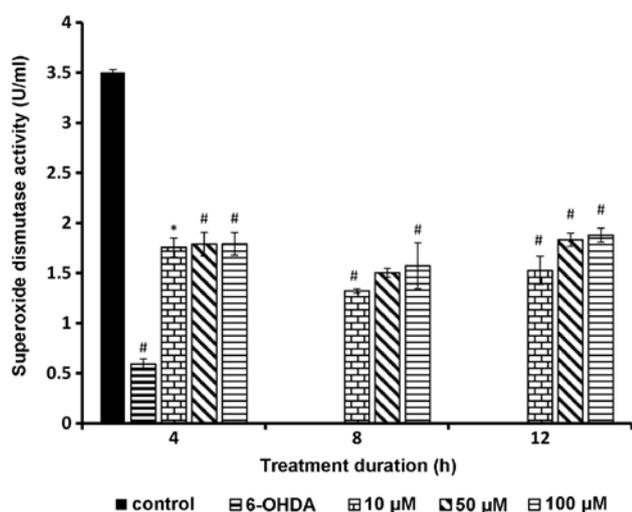
Figure 4. Total superoxide dismutase enzyme activity measured in rutin (10, 50 and 100 μM) pretreatment on 6-hydroxydopamine (6-OHDA) induced pheochromocytoma (PC-12) cells. Data are presented as mean ± SEM, (n=4) of three independent experiments. #P<0.01 and *P<0.05.
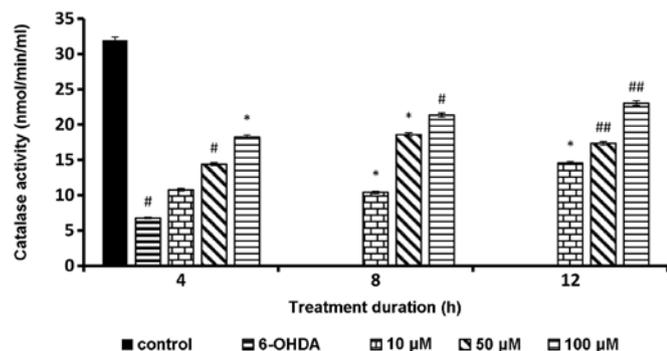

Figure 6. Time course of glutathione (GSH) concentration determined in three concentrations of rutin. Data are presented as mean ± SEM (n=4) of three independent experiments. #P<0.01.

Figure 5. The dose response of catalase enzyme activity quantitated in pretreatment of pheochromocytoma (PC-12) cells at 10, 50 and 100 μM of rutin. Results are presented as mean ± SEM (n=4) of three independent experiments. *P<0.05, #P<0.01 and ##P<0.001.

Figure 7. The endogenous glutathione (GSH) peroxidase released in response to three concentrations of rutin. Data are presented as mean ± SEM (n=4) of three independent experiments. *P<0.05.

GPx level following rutin pretreatment. GPx enzyme activity was determined colorimetrically. The GPx enzyme was significantly (P<0.05) higher in rutin pretreatment samples compared to the sample incubated with 6-OHDA alone. The enzyme activity was elevated at a low concentration of rutin and the data were statistically significant (P<0.01). This result demonstrated that PC-12 cells produced a lower amount of GPx in the presence of a high amount of external antioxidant (Fig. 7).

Lipid peroxidation following rutin pretreatment. A significant reduction of the MDA level was observed in cells treated with rutin (P<0.01). The cells treated with 6-OHDA alone showed a markedly high level of MDA, which is an indicator of lipid peroxidation or oxidative stress. Rutin significantly reduced the lipid peroxidation in a dose-dependent manner (P<0.01). The highest concentration of rutin showed the greatest potential in suppressing lipid peroxidation in PC-12 cells. The test groups exhibited a statistically significant decrease in lipid peroxidation compared to 6-OHDA treated cells alone (Fig. 8).
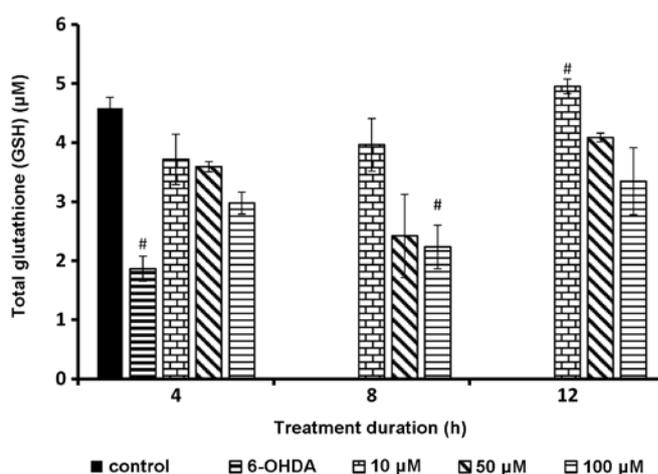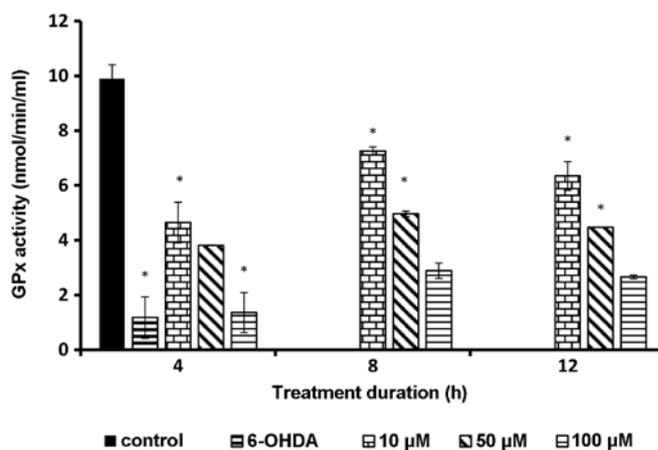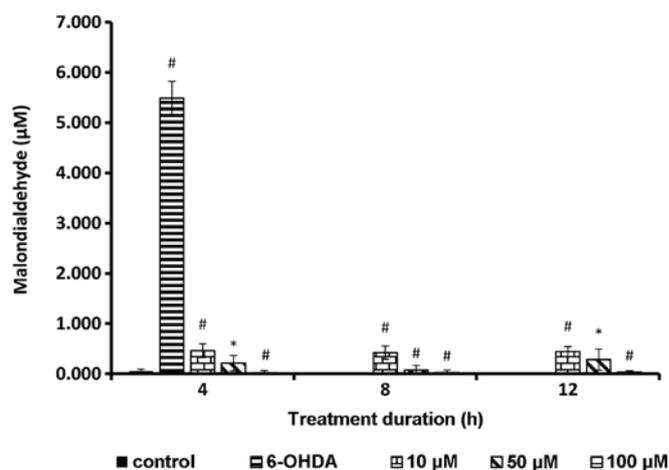

Figure 8. Effect of rutin pretreatment on lipid peroxidation caused by 6-hydroxydopamine (6-OHDA) on pheochromocytoma (PC-12) cells. Data are presented as mean ± SEM (n=4) of three independent experiments. *P<0.05 and #P<0.01.

Discussion

Reactive oxygen species (ROS) such as superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2) have been proven to be the major contributing factor in neurodegenerative diseases such as Parkinson's and Alzheimer's diseases (12). Parkinson's disease (PD) is caused by selective loss of neuronal cells in substantia nigra pars compacta, a region in the mid brain (13). Although various studies have been conducted to identify a neurotherapeutic agent for PD, an appropriate treatment regimen for PD remains elusive. Levodopa is considered the cornerstone treatment for PD. However, the efficacy of Levodopa tends to become depleted as the severity of disease progresses (11). Therefore, numerous attempts have been undertaken to identify a superior neurotherapeutic agent for PD.

Rutin, a quercetin glycoside compound is widely distributed in citrus fruits and buckwheat. The neuroprotective potential of rutin has been studied and documented (7). However, the literature regarding the neuroprotective effect of rutin is scanty. In this study, we have explored the role of the bioflavonoid antioxidant rutin on 6-OHDA-induced neurotoxicity in PC-12 cells. PC-12 cells were used as they resemble the neuronal cells at substantia nigra pars compacta in human brain (14). 6-OHDA is a neurotoxin that is usually used to induce oxidative stress in PC-12 cells. Subsequent to the pretreatment of PC-12 cells with rutin at 10, 50 and 100 μM for 4, 8 and 12 h, the cells were incubated with 6-OHDA for 24 h to induce oxidative stress. The cell viability assay and antioxidant enzyme assay were performed on the treated cells and cell lysate, respectively.

Results of the cytoprotective study showed that rutin markedly increased cell viability compared to cells with 6-OHDA alone. The cell viability increased significantly at 4 h of treatment in a dose-dependent manner, reaching the highest percentage at the 8th hour of pretreatment. The neuroprotective effect was directly proportional to the rutin concentrations. Aerobic cells are equipped with an extensive antioxidant enzyme network that eliminates ROS which was formed during intracellular signaling and defense against microorganisms (14). Imbalance in the ROS/antioxidant enzyme homeostasis may result in disease state (15).

The endogenous antioxidant enzyme levels were measured in all the samples that underwent rutin pretreatment and oxidative stress by 6-OHDA. The enzymes examined in this study were SOD, catalase, GSH, GPx and MDA concentrations. SOD enzymes are metalloenzymes that occur as Cu/Zn SOD, Mn-SOD and EC-SOD in aerobic cells. The enzymes are distributed in different compartments of cells, thus, Cu/Zn-SOD has been identified in cytosol, Mn-SOD in mitochondria and EC-SOD in the extracellular compartment of aerobic cells (16). The SOD enzyme occurs in a significantly high amount in brain, liver, heart, erythrocytes and kidney cells. The function among the three SODs is similar and involves catalyzing dismutation of the superoxide anion to oxygen molecules and hydrogen peroxide, a less toxic molecule (17). In this study, the total SOD enzyme was elevated in all the rutin pretreated cells in a dose-dependent manner. Elevation of the SOD enzyme as the rutin concentration increases proved that this antioxidant caused direct activation of SOD to catalyze O_2^- produced by 6-OHDA.

Catalase, a tetrameric enzyme is a ubiquitous enzyme identified in most aerobic cells that detoxifies hydrogen peroxide which was generated by SOD (2,3). Catalase converts two molecules of hydrogen peroxide to form two molecules of water and one molecule of oxygen. This catalytic reaction is a one-step process. In the present study, the catalase activity was elevated as the concentration of rutin increased from 10 to 100 μM. Therefore, rutin likely has a synergistic effect with catalase as it causes a direct activation of catalase by eliminating the ROS molecules from the system.

GPx, a selenocysteine antioxidant enzyme identified in the cytosol compartment of eukaryotic cells, is a vital antioxidant defense mechanism. GPx catalyzes the reduction of hydroperoxides at the expense of reduced GSH, a tripeptide to produce GSSG and reduced hydroperoxide. The GSSG is recycled to GSH by glutathione reductase and NADPH. In this study, the GPx and GSH levels were significantly increased in all the rutin-treated cells compared to 6-OHDA-treated cells alone. Findings showed that rutin interacted with GPx and GSH to exhibit its antioxidant mechanism. However, the trend seems to be inversely proportional to the rutin concentration as a large amount of hydroperoxides was already catalyzed by the enzyme catalase at high rutin concentrations, leading to a decreased amount of GPx, while GSH were activated to detoxify the hydroperoxides.

MDA is a naturally occurring end product of lipid peroxidation. Lipid peroxidation is a largely accepted concept of cell damage leading to disease onset. The antioxidant molecules protect the cells by reversing or suppressing the progression of lipid peroxidation in aerobic cells. Lipid peroxidation end products measured as thiobarbituric acid reactive substances were increased in the 6-OHDA-treated group. Rutin with antioxidant properties may provide endogenous defense systems and reduce both the initiation and propagation of reactive oxygen species. Rutin at different doses effectively reduced the increased levels of thiobarbituric acid reactive substances in treated cells.

The present findings demonstrated that rutin protects PC-12 neuronal cells against 6-OHDA-induced neurotoxicity. The drug may be considered as a potent therapeutic agent for neurodegeneration associated with free radical generation in the central nervous system. Additional experiments are required to clarify the mechanisms of this bioflavonoid. Studies are currently in progress to determine the molecular mechanisms of rutin-induced neuroprotection in PC-12 cells.

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