

# Quercetin exerts a neuroprotective effect through inhibition of the iNOS/NO system and pro-inflammation gene expression in PC12 cells and in zebrafish

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**Abstract.** Flavonoids have been reported to be potent antioxidants and beneficial in the treatment of oxidative stress-related diseases. Quercetin, a major flavonoid naturally occurring in plants, deserves attention because of its beneficial effects observed in various *in vitro* and *in vivo* neural damage models; however, the actions of quercetin are paradoxical. In an effort to confirm the neuroprotective effect of quercetin and to elucidate its mechanism of action, the neuroprotective effects of quercetin in PC12 cells and in zebrafish models were investigated. In this study, the selective dopaminergic neurotoxin 6-hydroxydopamine (6-OHDA), was used to induce neural damage in PC12 cells and zebrafish models. Pretreatment with quercetin offered neuroprotection against 6-OHDA-induced PC12 cell death. Moreover, quercetin could prevent 6-OHDA-induced PC12 cell apoptosis and 6-OHDA-stimulated dopaminergic neuron loss in zebrafish. Interestingly, quercetin was able to protect, but not rescue the dopaminergic neuron damage when zebrafish were treated with quercetin at different maturation stages of the blood brain barrier. A mechanistic study showed that quercetin could inhibit NO over-production and iNOS over-expression in PC12 cells and could down-regulate the over-expression of pro-inflammatory genes (e.g. IL-1 $\beta$ , TNF- $\alpha$  and COX-2) in zebrafish, suggesting that these genes play a role in the neuroprotective effect of quercetin. The objective of this study was to provide a scientific rationale for the clinical use of quercetin, leading to its development as an effective therapeutic agent for the treatment of Parkinson's disease.

## Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease with physiological manifestations including tremors, bradykinesia, abnormal postural reflexes, rigidity and akinesia. Although the etiology of PD is not completely understood, increasing evidence suggests that oxidative damage induced by reactive oxygen species (ROS) and reactive nitrogen species (RNS) is involved in the progression of dopaminergic neurodegeneration (1,2). As a result, inhibition of ROS and of nitric oxide (NO) and iNOS production, serve as potential targets for protection against PD pathogenesis.

Increasing evidence suggests that neuro-inflammatory processes contribute to the cascade leading to the progressive neuronal damage in PD (3). Activation of glial cells, such as astrocytes and microglia also plays a key role in the development of inflammatory neurodegeneration (4). The major intermediate of glial-induced neurotoxicity is the generation of NO via increased expression of iNOS. Excessive NO can induce neuronal cell damage by disrupting the function of the neuronal mitochondrial electron transport chain (5). Inflammation is also characterized by increased production of cytokines, such as IL-1 $\beta$  and TNF- $\alpha$ , which also act to stimulate iNOS and NO production (6). Hence, modulation of NO production, glial signalling cascades and pro-inflammatory cytokines might result in suppression of neuro-inflammation and may ultimately protect against neurodegeneration. Although the neuroprotective effects of quercetin are found to be via its anti-oxidative and anti-inflammatory capacity in an *in vitro* system of lipopoly-saccharide-activated co-culture of microglial and neuronal cells (7,8), it is uncertain whether its mode of action in the *in vivo* PD model involves anti-inflammation.

Quercetin, which is one of the most common naturally occurring flavonoids, has been shown to possess diverse biological activities, including anti-tumoral, anti-thrombotic, anti-inflammatory and anti-apoptotic effects (9-12). Although quercetin and structurally related flavonoids have been shown to have a neuroprotective capacity in various *in vitro* and *in vivo* experimental models (13-16), the neuroprotective

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effect of quercetin remains controversial (17). Nevertheless, quercetin did not protect *substantia nigra* neurons from an oxidative insult *in vivo*, probably due to its inability to cross the blood-brain barrier (BBB) under *in vivo* conditions (18). There is an urgent need for appropriate *in vivo* studies in order to confirm the neuroprotective effects of quercetin and to identify the reason for the discrepancy between earlier *in vitro* and *in vivo* findings.

The zebrafish (*Danio rerio*) has become a powerful model for drug discovery (19,20). Its rapid development and rather simple, short assay time make zebrafish a useful tool in large-scale drug screening (21). The brain structure and function of the zebrafish are very similar to those of other vertebrates (22). The anatomy of the zebrafish brain dopaminergic (DA) system was studied recently, and a region anatomically similar to the striatum was identified in the forebrain (23). Most neurotoxins, such as MPTP, 6-hydroxydopamine (OHDA) and rotenone, are known to induce DA neuron loss in animal models. Zebrafish PD models have been well validated and zebrafish develop relevant PD pathological conditions in response to these toxins (24–26). Moreover, exposure of zebrafish to neurotoxins induces relevant clinical behavioural features of PD reflected by reduced swimming velocity and total distance moved. In addition, those clinical and experimental neuroprotective agents have been demonstrated to have equivalent physiological activity in protecting zebrafish from neuronal insult (27,28). Taken together, the results of these earlier studies suggest that the zebrafish is a good alternative PD model and offers great opportunity for screening and discovery of novel PD therapeutic agents. In order to address the above-mentioned controversy concerning the use of quercetin for the treatment of PD and the lack of details about the anti-neuro-inflammatory action of quercetin *in vivo*, the neuroprotective effect and the underlying mechanism of action of quercetin were investigated using 6-OHDA-treated zebrafish and PC12 cells in this study.

## Materials and methods

**Cell culture.** Stock cultures of rat pheochromocytoma cells (PC12) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). They were cultured in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C in F-12K medium supplemented with 15% (v/v) heat-inactivated horse serum, 2.5% (v/v) FBS, penicillin (100 U/ml) and streptomycin (100 µg/ml), which was changed every other day.

**3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay.** MTT is a tetrazolium salt that can be reduced to purple formazan by living cells. Cells were incubated for 4 h at 37°C in MTT solution (prepared in fresh 0.5% (v/v) heat-inactivated horse serum; final concentration 0.5 mg/ml). The medium was then discarded and 150 µl of DMSO was added to the well to dissolve the violet formazan crystals in intact cells. The absorbance was measured at a wavelength of 490 nm by a multilabel counter (Wallac Victor3™ V, Perkin-Elmer, The Netherlands). Cell viability was expressed as a percentage of the control (untreated cells). All assays were done in 8 replicates and at least 3 times.

**Lactate dehydrogenase (LDH) assay.** Cell viability was determined by measuring the activity of LDH released into the incubation medium when cellular membranes are damaged. Cells were plated in 96-well plates and the amount of LDH released was measured and then the cells were lysed to measure the total LDH. Total and released LDH activities were determined according to the instructions accompanying the Cytotoxicity Detection Kit (Roche). Absorbance was measured with an automatic microplate reader at 490 nm. LDH released was normalized to total LDH and the results are shown as a percentage of the control value.

**Intracellular NO detection.** Intracellular NO was evaluated by using the fluorescent probe 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM diacetate), which is cell-permeant and diffuses passively across cellular membranes. Once inside the cells, DAF-FM diacetate is deacetylated by intracellular esterases to DAF-FM, which is essentially non-fluorescent until it reacts with NO; thus, it can be used to quantify intracellular NO production. The cells were seeded in 96-well, black bottom-clear plates. After treatment with quercetin for 6 h and then exposure to 6-OHDA for 1 h, cells were washed in PBS and incubated for 20 min at 37°C in darkness in a medium containing 1% serum plus 2.5 µM DAF-FM diacetate. The cells were then washed twice in PBS and the fluorescence was evaluated in a microplate reader at an excitation wavelength of 495 nm and an emission wavelength of 515 nm. The increase in fluorescence for each treatment was calculated as the relative fluorescence of each treatment compared with the untreated control cells.

**Western blot analysis.** After treatment, PC12 cells were washed three times with PBS and then RIPA lysis buffer containing 1% PMSF and 1% Protease Inhibitor Cocktail and incubated for 30 min on ice. Cell lysates were centrifuged at 12,500 × g for 20 min at 4°C. The supernatant was separated and the amount of protein was determined using the BCA protein assay kit. Protein samples (30 µg) were separated by SDS-PAGE (12% (w/v) polyacrylamide gel) and then transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA). Subsequently, the membrane was blocked with 5% (v/v) non-fat milk in PBST (PBS containing 0.1% Tween-20) for 1 h at room temperature. The blots were incubated overnight at 4°C with primary antibodies anti-iNOS and anti-β-actin (1:1000, from Cell Signaling, Inc.). β-actin was used as the internal reference. After three washes with PBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000) in PBST with 5% non-fat milk for 1 h at room temperature. After repeated washes, proteins were visualized with an ECL advanced Western blotting detection kit (Amersham, UK) according to the manufacturer's protocol. Photographs of protein bands were taken by the Molecular Imager ChemiDoc XRS (Bio-Rad). Quantitative assessment of protein bands was done with Gel Doc™ XRS equipped with Quantity One Software.

**Fish maintenance.** The AB wild-type strain of zebrafish was used for this study. Embryos were collected after natural spawning, staged according to standard criteria, and raised

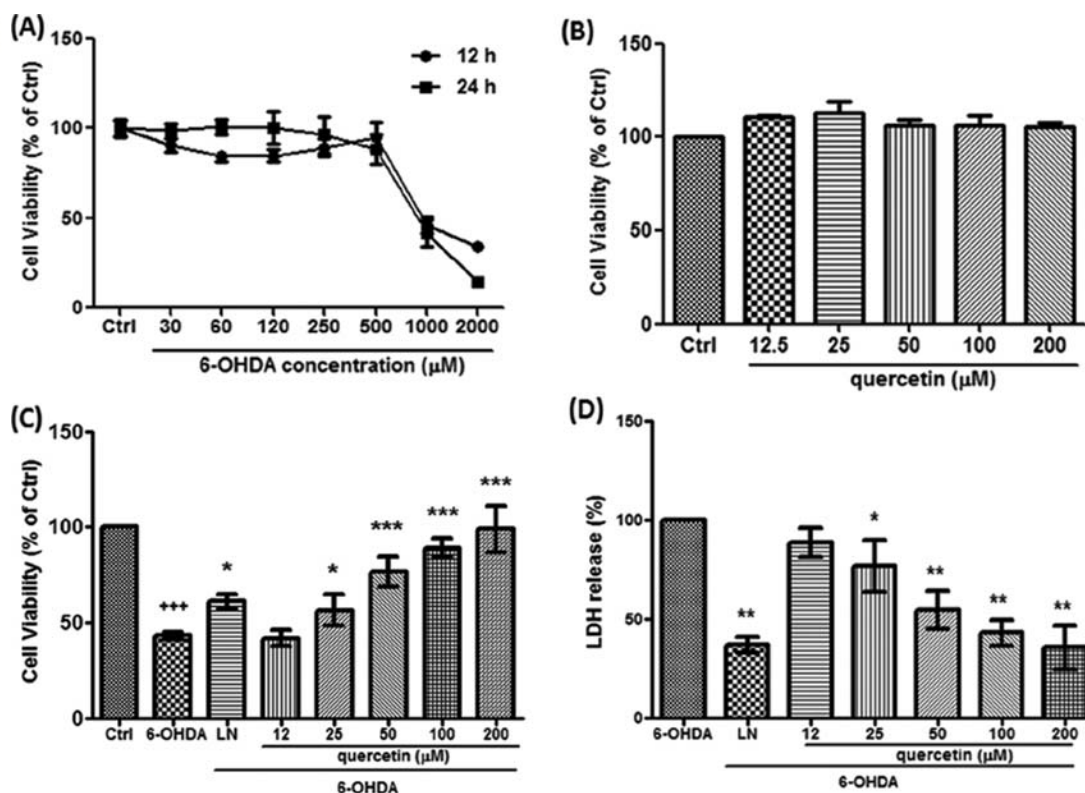


Figure 1. Quercetin dose-dependent inhibition of 6-hydroxydopamine (6-OHDA)-induced cell death in PC12 cells. (A) PC12 cells were incubated for 12 h or 24 h with different concentrations of 6-OHDA. (B) Cells were treated for 6 h with or without different concentrations of quercetin. (C, D) Cells were pretreated for 6 h with or without different concentrations of quercetin. Cells pretreated for 6 h with 250  $\mu\text{M}$  L-NAME (LN) served as a positive control. All groups were exposed to 1 mM 6-OHDA for another 12 h after the pretreatment. In A-C, cell viability was measured by the MTT assay and the results are expressed as a percentage of that of the control group (without treatment with 6-OHDA). (D) Same as (C) and (D), except the degree of cell death was measured as the percentage of released lactate dehydrogenase (LDH). \*\*\* $p < 0.0001$  vs. the control group; \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.0001$  vs. the 6-OHDA group. Each experiment in the figure was done at least in triplicate.

synchronously at 28.5°C in embryo medium (13.7 mM NaCl, 540  $\mu\text{M}$  KCl, pH 7.4, 25  $\mu\text{M}$   $\text{Na}_2\text{HPO}_4$ , 44  $\mu\text{M}$   $\text{KH}_2\text{PO}_4$ , 300  $\mu\text{M}$   $\text{CaCl}_2$ , 100  $\mu\text{M}$   $\text{MgSO}_4$ , 420  $\mu\text{M}$   $\text{NaHCO}_3$ , pH 7.4). No additional maintenance was required because the embryos receive nourishment from the attached yolk ball. Ethical approval for the animal experiments was granted by the Animal Research Ethics Committee, University of Macau.

**Anti-tyrosine hydroxylase (TH) whole-mount immunostaining.** Zebrafish were fixed in 4% paraformaldehyde in PBS for 5 h, rinsed, and stored at -20°C in 100% EtOH. Whole-mount immunostaining was done by standard methods (29). Briefly, fixed samples were blocked (2% lamb serum and 0.1% BSA in PBST) for 1 h at room temperature. A mouse monoclonal anti-TH antibody (1:200 diluted in blocking buffer, MAB318, Millipore) was used as the primary antibody and incubated with the sample overnight at 4°C. The next day, samples were washed 6 times with PBST (30 min each wash), followed by incubation with secondary antibody according to the instructions of the Vectastain ABC kit (Vector Laboratories, Inc.). After staining, zebrafish were flat-mounted with 3.5% methylcellulose and photographed.

**Total RNA extraction and reverse transcription and quantitative real-time PCR.** Zebrafish embryos at 48 hpf were treated for 24 h with different concentrations of quercetin in the presence or absence of 6-OHDA. Total RNA was extracted

from 40 zebrafish embryos of each treatment group using the RNeasy Mini Kit (Qiagen, USA) in accordance with the manufacturer's instructions. RNA was reverse transcribed to single-stranded cDNA using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, USA), followed by real-time PCR using the TaqMan® Universal PCR Master Mix and TaqMan® gene expression assay primer for zebrafish IL-1 $\beta$  (assay ID Dr03114368\_m1), TNF- $\alpha$  (assay ID Dr03126849\_g1) and COX-2 (assay ID Dr03080323\_m1) in the ABI 7500 real-time PCR system (all products from Applied Biosystems). The expression level of each gene was expressed as the relative fold change calculated using the comparative CT method (30) and  $\beta$ -actin was used as the internal reference.

**Statistics.** One-way ANOVA and the Dunnett's test were used to identify significant differences between treatment groups and the vehicle control. All calculations were done using the GraphPad Prism statistical software (GraphPad Software, Inc., San Diego, CA) and the level of statistical significance was set at  $p < 0.05$ .

## Results

**Quercetin protects PC12 cells against 6-OHDA-induced cytotoxicity.** 6-OHDA-induced cellular damage was evaluated by determining the percentage of MTT reduction in PC12



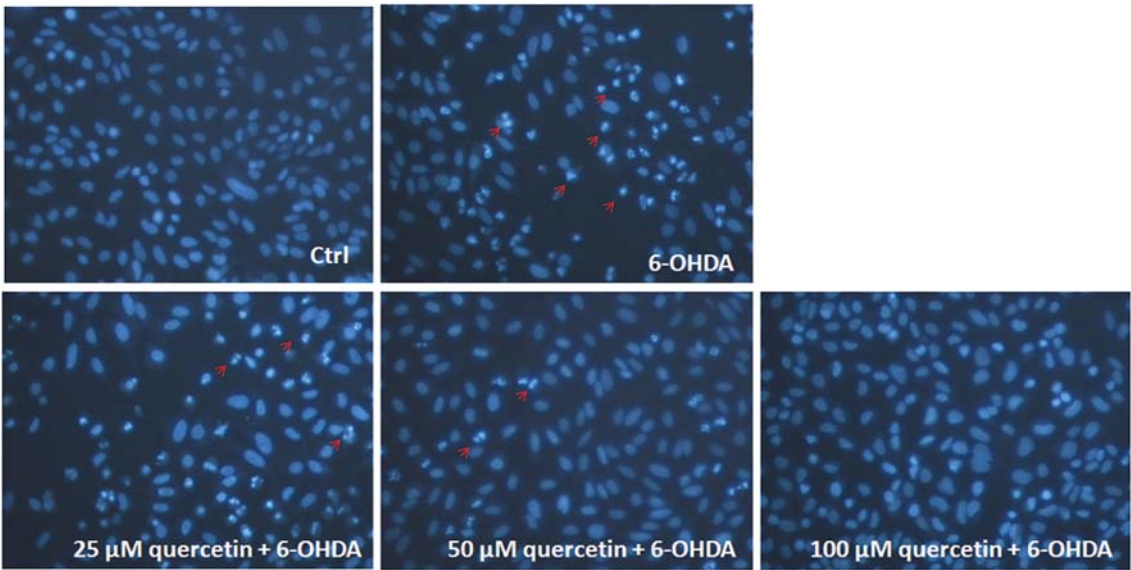


Figure 2. Anti-apoptotic effect of quercetin against 6-hydroxydopamine (6-OHDA)-induced damage to PC12 cells. PC12 cells were treated for 6 h with or without different concentrations of quercetin, then exposed to 1 mM 6-OHDA for another 12 h. Cells were stained with the DNA-binding fluorescent dye Hoechst 33342. Chromatin condensation and DNA fragments are indicated by the red arrows. Magnification x400.

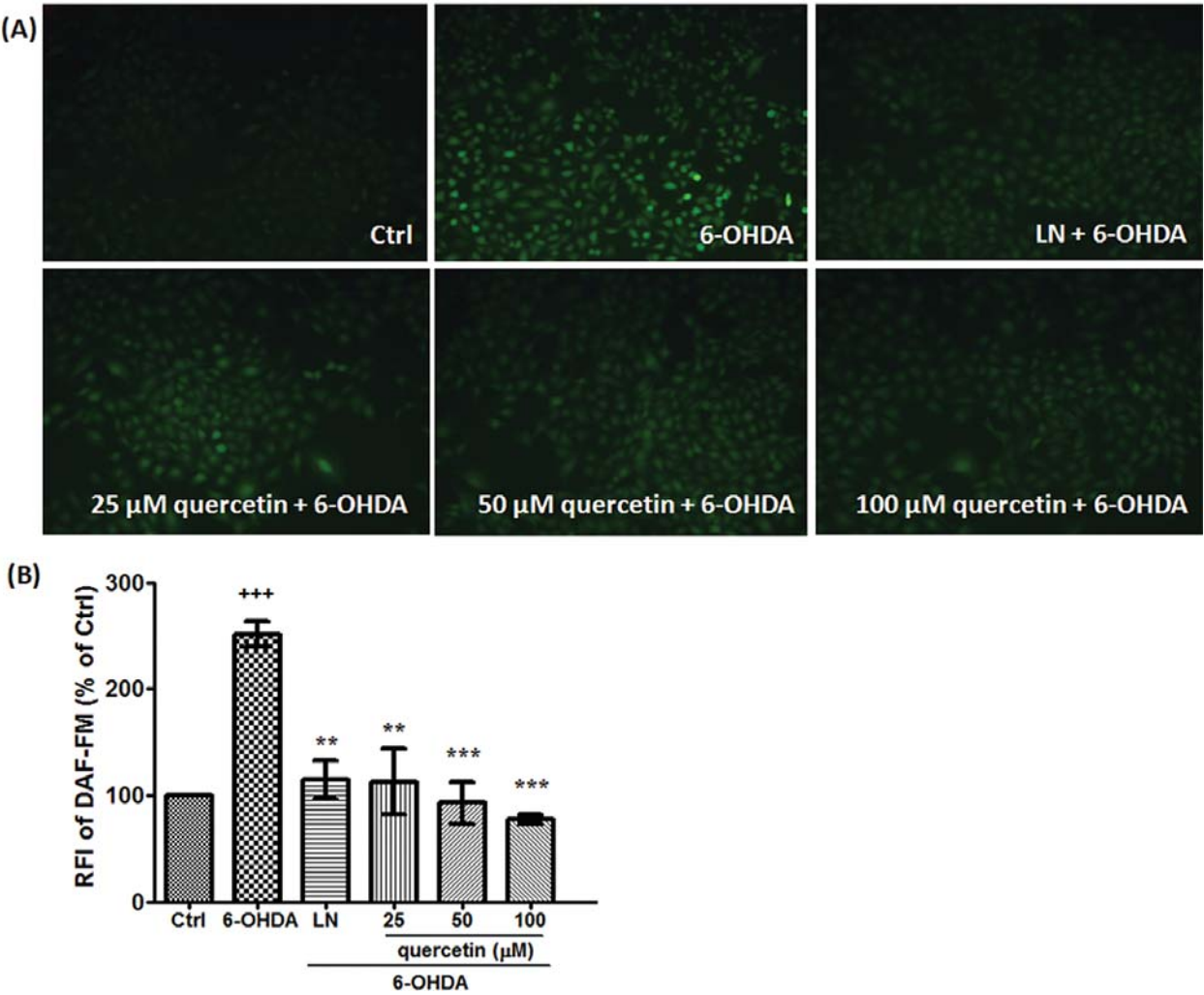


Figure 3. Quercetin-inhibited 6-hydroxydopamine (6-OHDA)-induced nitric oxide (NO) over-production in PC12 cells. PC12 cells were treated for 6 h with or without different concentrations of quercetin, then exposed to 1 mM 6-OHDA for 1 h. (A) Intracellular NO was stained by the fluorescent indicator DAF-FM diacetate. (B) The NO fluorescent intensity was quantified by a multilabel counter. Results are expressed as a percentage of relative fluorescent intensity (RFI) of the control (Ctrl) group. \*\*\* $p < 0.0001$  vs. the control group; \*\* $p < 0.01$  and \*\*\* $p < 0.0001$  vs. the 6-OHDA group. Magnification x200.



shown in Fig. 1A, 6-OHDA caused a dose-dependent reduction of PC12 cell viability. When PC12 cells were exposed to 1 mM 6-OHDA for 12 h, cell viability was reduced significantly to  $46.2 \pm 4.5\%$  of the control group viability. Cytotoxicity evaluation indicated that quercetin did not impose any cytotoxicity on PC12 cells up to the maximum concentration of 200  $\mu\text{M}$  (Fig. 1B). In order to study the neuroprotective effects of quercetin, PC12 cells were treated with different concentrations (12.5, 25, 50, 100 and 200  $\mu\text{M}$ ) of quercetin for 6 h before exposure to 1 mM 6-OHDA for 12 h. The protective effects of quercetin were determined by the MTT and LDH assays. As shown in Fig. 1C, pretreatment with quercetin protected PC12 cells against 6-OHDA-induced damage in a dose-dependent manner. Similarly, pretreatment with quercetin significantly reduced ( $p < 0.05$ ) the LDH leakage caused by 6-OHDA (Fig. 1D). L-NAME (LN) is an iNOS inhibitor that protects PC12 cells against 6-OHDA cytotoxicity by inhibiting 6-OHDA-induced NO over-production and served as the positive control in this study (31). In the present study, the protective effect of quercetin was even stronger than that of LN.

**Quercetin protects PC12 cells against 6-OHDA-induced apoptosis.** In order to investigate whether 6-OHDA induces cell death through apoptosis, DNA staining with Hoechst 33342 was used to evaluate chromatin condensation and nuclear fragmentation in PC12 cells. As shown in Fig. 2, normal cells were circular or elliptical with no nuclear condensation or fragmentation. In contrast, bright condensed dots known as apoptotic bodies (Fig. 2, red arrows) were clearly identified in 6-OHDA-treated PC12 cells and reductions in colony density and cell size were observed. However, 6-OHDA-induced nuclear condensation was attenuated significantly by pretreatment with quercetin (Fig. 2, lower panel). Moreover, higher quercetin concentrations (50 and 100  $\mu\text{M}$ ) inhibited 6-OHDA-induced colony reduction and cell shrinkage.

**Quercetin attenuates 6-OHDA-induced NO over-production and iNOS over-expression in PC12 cells.** Augmented NO production subsequent to iNOS induction appears to play an important role in the initial phase of 6-OHDA-induced neuro-damage models *in vitro* and *in vivo* (32-34). We tested the effect of quercetin on 6-OHDA-induced NO over-production and iNOS over-expression in PC12 cells. Stimulation of PC12 cells with 1 mM 6-OHDA for 1 h caused a significant, almost 3-fold, increase compared to untreated cells (Fig. 3). Because 6-OHDA-induced NO over-production decreased when treatment with 6-OHDA was for longer than 1 h (data not shown), we incubated PC12 cells with 6-OHDA for 1 h when investigating the NO-inhibitory effect of quercetin. As shown in Fig. 3A and B, pretreatment with quercetin for 6 h inhibited 6-OHDA-induced NO over-production in a concentration-dependent manner and the level of NO was attenuated to normal (control group with no 6-OHDA treatment) by high concentrations (50 and 100  $\mu\text{M}$ ) of quercetin. Moreover, the NO-inhibitory effect of high concentrations of quercetin (50 and 100  $\mu\text{M}$ ) was stronger than that in the positive control, which used 250  $\mu\text{M}$  LN, a non-selective iNOS inhibitor (Fig. 3). These results are, in

part, supportive of our finding shown in Fig. 1, that quercetin exhibits stronger protective effects compared to LN against 6-OHDA-induced damage in PC12 cells.

Furthermore, Western blot analysis revealed that stimulation of PC12 cells with 6-OHDA for 30 min and 1 h significantly enhanced iNOS protein expression (Fig. 4A and C). However, the enhanced iNOS expression started to diminish when the cells were treated with 6-OHDA for longer than 1 h. The time-dependent response of iNOS expression to treatment with 6-OHDA was correlated with that of NO production (Fig. 3). Pretreatment with quercetin also results in a concentration-dependent down-regulation of 6-OHDA-induced iNOS over-expression, similar to the inhibitory effect observed in 6-OHDA-induced NO over-production. This implies that quercetin attenuated NO over-production via down-regulation of iNOS over-expression in 6-OHDA-treated PC12 cells.

**Quercetin can prevent, but not rescue the DA neuron loss induced by 6-OHDA in zebrafish.** The development of DA neurons in zebrafish is similar to that in other vertebrates and thus the zebrafish is a good model to study disorders of the DA system (23). To further investigate the neuroprotective effect of quercetin *in vivo*, we examined the DA neurons in zebrafish by whole-mount immunostaining with an antibody against TH, the rate-determining enzyme involved in the synthesis of DA. As shown in Fig. 5B and C, the diencephalic DA neurons became matured at 3 dpf. When compared with the control, exposure of 1 dpf zebrafish to 6-OHDA for 48 h results in about 50% reduction of TH-positive neurons in the diencephalic area of the zebrafish embryo. Co-treatment with quercetin (6 or 12  $\mu\text{M}$ ) significantly inhibited 6-OHDA-induced DA neuron loss by approximately 20% compared with the 6-OHDA group (Fig. 5B). Nomifensine, a DAT inhibitor, was used as the positive control and was shown to exert considerable protection against 6-OHDA-induced DA neuron loss (Fig. 5B). However, quercetin could not reverse the 6-OHDA-induced DA neuron loss if it was administered after a 48 h exposure to 6-OHDA (Fig. 6).

**Quercetin reverses 6-OHDA-induced pro-inflammatory gene over-expression in zebrafish.** Neuro-inflammation plays a key role in 6-OHDA-induced DA neuron damage *in vivo* (35). We used quantitative real-time PCR to measure inflammation-related gene expression in 6-OHDA-treated zebrafish. As shown in Fig. 7, 6-OHDA caused over-expression of IL-1 $\beta$ , TNF- $\alpha$  and COX-2, several fold greater than that of untreated control fish. Co-treatment with quercetin reversed the up-regulated inflammatory gene expression, revealing that the anti-inflammatory activity of quercetin is involved in its neuroprotective effect in the zebrafish model of 6-OHDA-induced neuronal damage.

## Discussion

The present study showed that quercetin is a potent neuroprotective agent in both PC12 cells *in vitro* and zebrafish *in vivo*. Furthermore, we have shown that its neuroprotective activity might be exerted via the inhibition of NO over-production by down-regulating the over-expression of iNOS

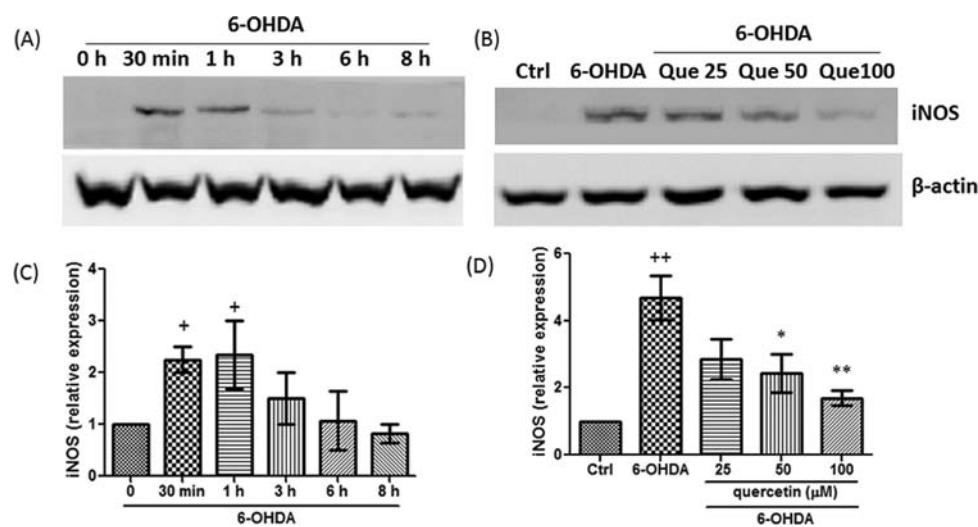


Figure 4. Quercetin down-regulates iNOS over-expression in PC12 cells stimulated by 6-hydroxydopamine (6-OHDA). (A, C) PC12 cells were treated with 1 mM 6-OHDA for 30 min, 1, 3, 6 and 8 h. iNOS expression was evaluated at these time points. (B, D) PC12 cells were treated for 6 h with quercetin (Que), then exposed to 1 mM 6-OHDA for another 1 h. Quercetin dose-dependent inhibition of 6-OHDA-induced iNOS over-expression. <sup>+</sup>*p*<0.05 vs. 0 h group (before treatment with 6-OHDA); <sup>++</sup>*p*<0.01 vs. the control group; <sup>\*</sup>*p*<0.05 and <sup>\*\*</sup>*p*<0.01 vs. the 6-OHDA-treated group.

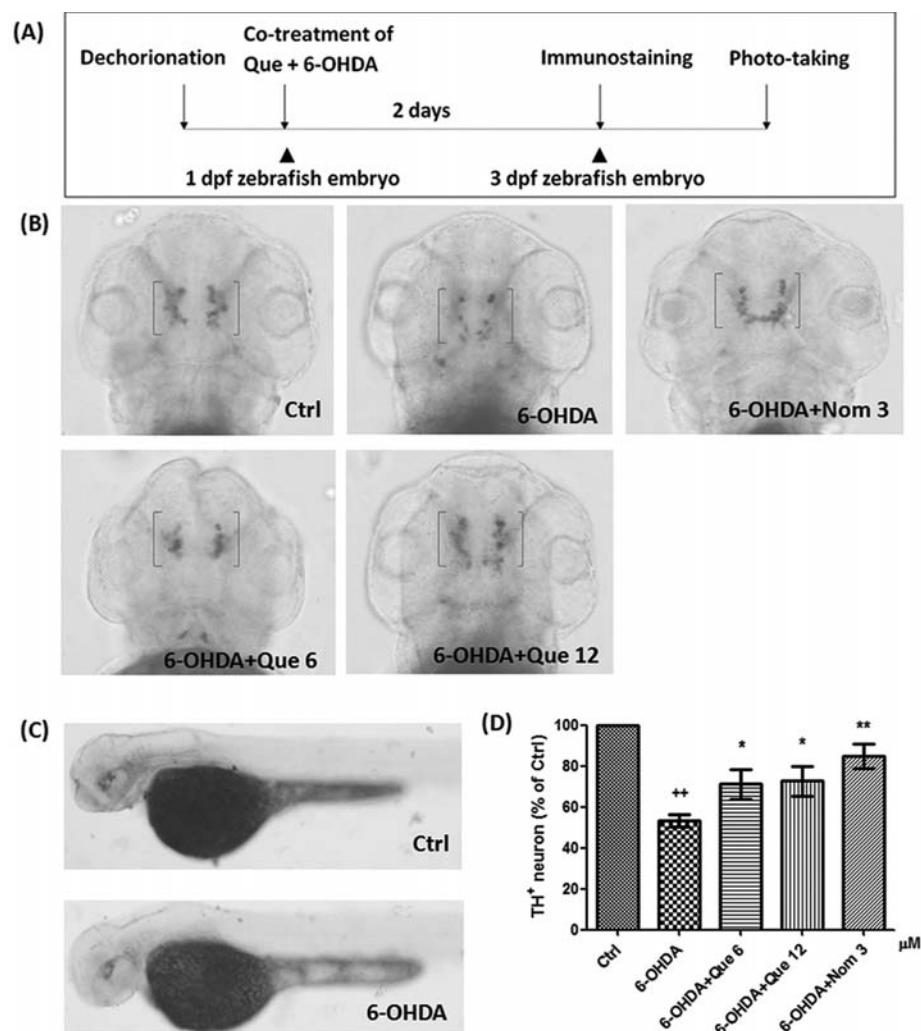


Figure 5. Quercetin protects against the 6-hydroxydopamine (6-OHDA)-induced dopaminergic neuron loss in zebrafish. (A) A schematic illustration of drug treatment. 1 dpf dechorionated zebrafish were treated for 48 h with 250  $\mu$ M 6-OHDA in the presence or absence of quercetin (Que) or nomifensine (Nom, a DAT inhibitor used as a positive control). After treatment, anti-tyrosine hydroxylase (TH) immunostaining was used to detect the DA neurons in zebrafish. (B and C) Representative morphology of DA neurons in zebrafish brain. The diencephalic DA neurons (Di DA) are indicated by brackets. (B) Ventral view, head at the top; (C) lateral view, head at the left. (D) Quantitative analysis of TH<sup>+</sup> neurons in zebrafish brain. Data are expressed as a percentage of the control group. Each bar represents mean  $\pm$  SEM. <sup>++</sup>*p*<0.001 vs. the control group; <sup>\*</sup>*p*<0.05 vs. and <sup>\*\*</sup>*p*<0.01 vs. the 6-OHDA-treated group.



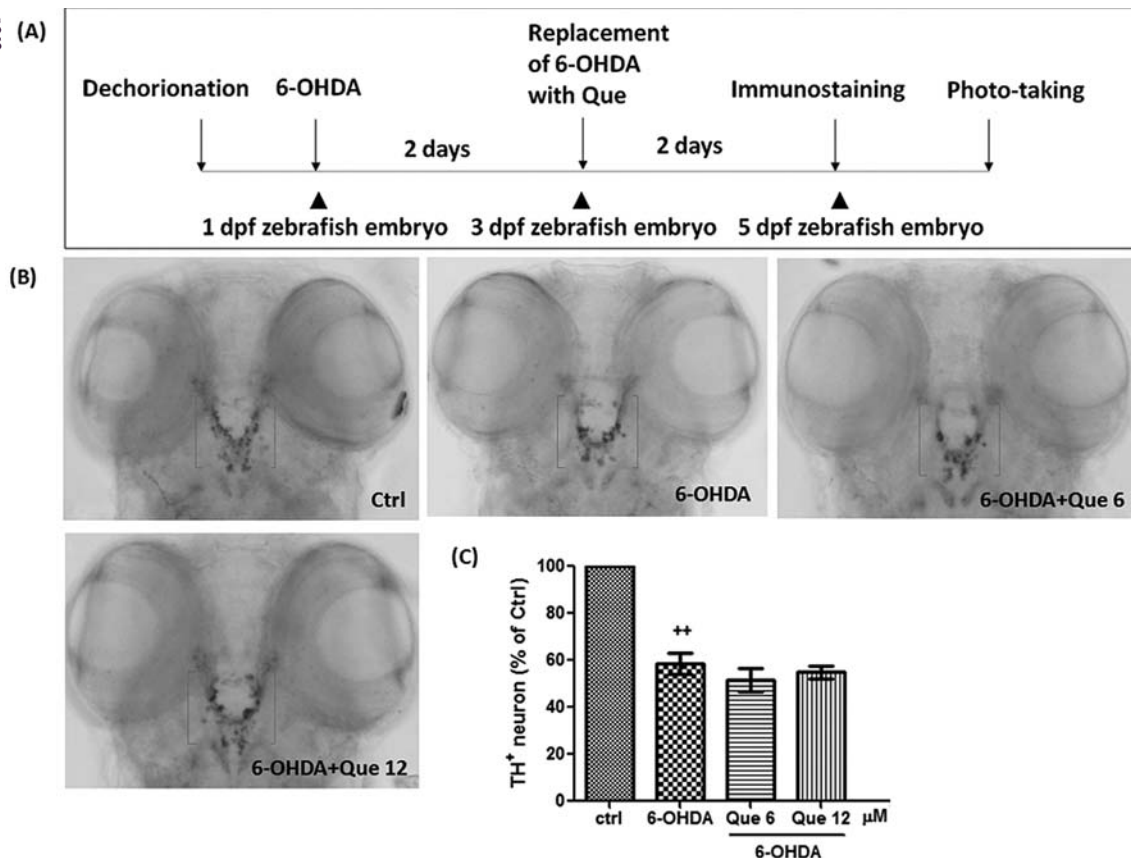
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Figure 6. Quercetin (Que) did not rescue DA neuron loss post-6-hydroxydopamine (6-OHDA) treatment in zebrafish. (A) A schematic illustration of drug treatment. Dechorionated 1 dpf zebrafish were treated for 48 h with 250  $\mu$ M 6-OHDA, then 6-OHDA was replaced with Que and treatment was continued for another 48 h. (B) Representative morphology of diencephalic DA neurons (Di DA) in zebrafish brain, indicated by brackets. (C) Quantitative analysis of tyrosine hydroxylase (TH)<sup>+</sup> neurons in zebrafish brain. Data are expressed as a percentage of the control (Ctrl) value. Each bar represents mean  $\pm$  SEM. <sup>++</sup>p<0.001 vs. the control group.

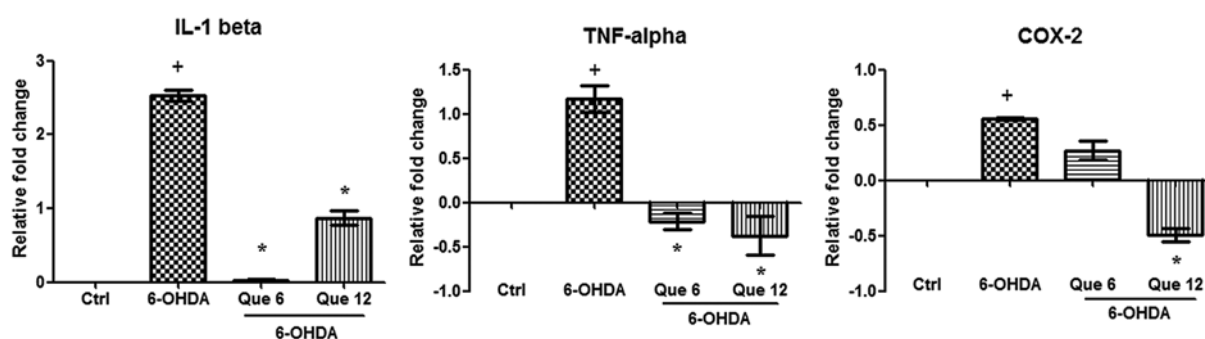


Figure 7. Quercetin (Que) down-regulates pro-inflammatory gene over-expression in zebrafish stimulated by 6-hydroxydopamine (6-OHDA). Zebrafish embryos (2 dpf) were treated for 24 h with 6-OHDA in the absence or presence of different concentrations of Que. After treatment, mRNA was extracted from each group; quantitative real-time PCR analysis of pro-inflammatory genes (IL-1 $\beta$ , TNF- $\alpha$  and COX-2) was performed. The results are expressed as the fold-change calculated by the relative Ct method using  $\beta$ -actin as the internal reference. Each bar represents mean  $\pm$  SEM. <sup>+</sup>p<0.05 vs. the control (Ctrl) group; <sup>\*</sup>p<0.05 vs. the 6-OHDA-treated group.

in PC12 cells and by the inhibiting inflammatory genes in zebrafish, respectively. To the best of our knowledge, this is the first study to demonstrate the neuroprotective and anti-inflammatory action of quercetin in zebrafish.

Our *in vitro* study showed that pretreatment with quercetin significantly protected PC12 cells against 6-OHDA neurotoxicity (Figs. 1 and 2), which is in accord with earlier reports

that quercetin has a protective effect against toxicity induced by MPP<sup>+</sup> (36) and H<sub>2</sub>O<sub>2</sub> (37) in PC12 cells. However, it has been reported that quercetin alone caused apoptosis and/or cell death in various cell types, including cancer (9) and neuronal SH-SY5Y (38) cells. In PC12 cells, rather than inhibiting, quercetin enhanced H<sub>2</sub>O<sub>2</sub>-induced PC12 cell death (39). Some effects of quercetin related to enhanced cell death are mediated by mechanisms that require longer drug

treatment to be manifested, such as by inhibiting heat shock protein 70 expression (40) or reducing the activation of the phosphoinositide 3-kinase pathway (41). A recent report illuminates a dual effect of quercetin from another point of view: quercetin might well be neuroprotective, but it has toxic metabolites that counteract this activity (42). It seems evident that quercetin regulates cell survival and cell death in different ways. For example, the protective effect of quercetin might be due to its anti-oxidative and ROS-scavenging properties, which would take place within a few minutes after the exposure of cells to 6-OHDA, but the toxic effects of the drug need more time to develop.

6-OHDA is selectively taken up by the plasma membrane dopamine transporter and subsequently accumulates in the mitochondria (43). Exposure to 6-OHDA results in the formation of ROS and RNS. It has been demonstrated that the oxidative damage induced by 6-OHDA could be prevented by treatment with antioxidants, such as melatonin (44,45). The results of the present study demonstrate that exposure to 6-OHDA significantly increases NO production and iNOS expression in PC12 cells. LN, a non-specific iNOS inhibitor, showed a neuroprotective effect in the 6-OHDA-induced cell damage in PC12 cells (Figs. 3 and 4). Since quercetin reduced NO over-production and down-regulated the over-expression of iNOS induced by 6-OHDA in PC12 cells, this finding suggests that quercetin exerts its neuro-protective effect through the iNOS-NO pathway.

This gene expression study in zebrafish (Fig. 7) indicated that 6-OHDA up-regulated expression of pro-inflammatory genes, such as IL-1 $\beta$ , TNF- $\alpha$  and COX-2. IL-1 $\beta$  and TNF- $\alpha$  are important pro-inflammatory cytokines, and COX-2 is a pro-inflammatory enzyme that mediates the production of prostaglandins; they all play important roles in the etiology of Parkinson's disease (46). It has been demonstrated that COX-2 is up-regulated in *substantia nigra* DA neurons in PD patients and in animal models (47). The inhibition of COX-2 and TNF- $\alpha$  has provided neuroprotection in rats (46). In this study, co-treatment with quercetin significantly suppressed pro-inflammatory gene over-expression stimulated by 6-OHDA. Thus, the neuroprotective effect of quercetin in the 6-OHDA-induced zebrafish model could be explained, at least in part, by its down-regulation of pro-inflammatory gene expression.

The *in vivo* neuroprotective effect of quercetin has been controversial for decades (17). Several earlier studies found that quercetin has no consistent neuroprotective effect in rat models of 6-OHDA-induced Parkinson's disease (18,38,48). In contrast, some studies showed that quercetin has beneficial effects in various *in vivo* models of cerebrovascular insults, possibly due to the increased permeability of the BBB in these animal models (49), which can facilitate delivery of quercetin into brain. In addition, treatment of permanent focal ischemia with a lecithin/quercetin preparation decreased lesion volume, showing that this pharmaceutical preparation dosage form could help quercetin to cross the BBB *in vivo* (37). These studies show that quercetin itself might cross the BBB ineffectively, which could possibly be enhanced by modified pharmaceutical formulation. Our *in vivo* study in zebrafish shows that when quercetin is administered at different maturation stages of the BBB in zebrafish it can

prevent, but not rescue, the 6-OHDA-induced DA neuron loss (Figs. 5 and 6). This phenomenon could be attributed to the poor permeability of quercetin across the BBB. The zebrafish is a recognized model for the analysis of basic developmental processes, and earlier studies demonstrated that the BBB of zebrafish is present by 72 hpf (28,50,51). Thus, when quercetin was administered to zebrafish larvae before 3 dpf, it could penetrate the brain rapidly and exert a protective effect against 6-OHDA toxicity because the BBB was not yet functionally well-developed. However, when quercetin was administered to zebrafish after 3 dpf, the matured BBB could pose an obstacle to quercetin reaching the brain, preventing it from rescuing the 6-OHDA-induced dopaminergic insult.

In conclusion, the results from the *in vitro* and *in vivo* assays in this study demonstrate the neuro-protective effects of quercetin and add insight into its mechanism of action in 6-OHDA-induced cytotoxicity and neurotoxicity. We have shown that both the iNOS-NO inhibitory and the anti-inflammatory actions of quercetin play key roles in the neuro-protective effect of quercetin. Also, the *in vivo* zebrafish data reported here demonstrate that quercetin can prevent, but not rescue the DA neuron loss induced by 6-OHDA at different maturation stages of the zebrafish BBB. Furthermore, the data provide insight into the importance of BBB permeability in the manifestation of the beneficial effects of quercetin in PD *in vivo*.


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