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Research Article

Quercetin and Sesamin Protect Dopaminergic Cells from MPP$^+$-Induced Neuroinflammation in a Microglial (N9)-Neuronal (PC12) Coculture System

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A growing body of evidence indicates that the majority of Parkinson's disease (PD) cases are associated with microglia activation with resultant elevation of various inflammatory mediators and neuroinflammation. In this study, we investigated the effects of 2 natural molecules, quercetin and sesamin, on neuroinflammation induced by the Parkinsonian toxin 1-methyl-4-phenylpyridinium (MPP$^+$) in a glial-neuronal system. We first established that quercetin and sesamin defend microglial cells against MPP$^+$-induced increases in the mRNA or protein levels of 3 pro-inflammatory cytokines (interleukin-6, IL-1β and tumor necrosis factor-alpha), as revealed by real time-quantitative polymerase chain reaction and enzyme-linked immunoabsorbent assay, respectively. Quercetin and sesamin also decrease MPP$^+$-induced oxidative stress in microglial cells by reducing inducible nitric oxide synthase protein expression as well as mitochondrial superoxide radicals. We then measured neuronal cell death and apoptosis after MPP$^+$ activation of microglia, in a microglial (N9)-neuronal (PC12) coculture system. Our results revealed that quercetin and sesamin rescued neuronal PC12 cells from apoptotic death induced by MPP$^+$ activation of microglial cells. Altogether, our data demonstrate that the phytoestrogen quercetin and the lignan sesamin diminish MPP$^+$-evoked microglial activation and suggest that both these molecules may be regarded as potent, natural, anti-inflammatory compounds.

1. Introduction

Parkinson's disease (PD) is a progressive, neurodegenerative disorder characterized by the loss of dopaminergic (DAergic) neurons in the *substantia nigra* (SN) and glial dysfunction. A new flow of information indicates that inflammation-derived oxidative stress and cytokine-dependent toxicity contribute to nigrostriatal pathway degeneration [1–3]. *Postmortem* studies have shown that microglia are activated regionally in the SN of PD patients as well as in PD animal models [4–6]. Microglia, resident immune cells of the brain, are activated in response to initiation factors (i.e., toxins, bacteria or viruses, pesticides, neuronal injury, etc.). These factors may also trigger a self-perpetuating cycle of chronic neuroinflammation, increasing the release of inflammatory chemical substances and promoting microglia activation. Besides, the SN is the brain region with the highest density of microglial cells [7]; thus, the neurons of this region are particularly susceptible to microglial-mediated toxicity *in vitro* and *in vivo* [8].

Proinflammatory cytokines and prostaglandins, identified in the SN, striatum and cerebrospinal fluid of PD patients *postmortem*, include tumor necrosis factor-alpha (TNFα), interleukin-1beta, -2, and -6 (IL-1β, IL-2, IL-6) [9, 10]. TNFα, IL-1α, IL-1β, and IL-6 have also been identified in PD animal models [11–13].

MPP$^+$ (1-methyl-4-phenylpyridinium), the active neurotoxic metabolite of the Parkinsonian toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), inhibits complex I of the mitochondrial respiratory chain, inducing energy depletion and producing reactive oxygen species (ROS), such as superoxide anion ($^{\bullet}O_2^-$) [14]. The latter can react with nitric oxide (NO) to generate the potent oxidant

peroxynitrite, which has been implicated in the development of several neurological diseases [15, 16]. Accumulation of activated microglia around DAergic neurons has been found in *postmortem* human brains with MPTP-induced parkinsonism [17]. In addition, MPTP primate models confirm that serum TNFα levels are elevated without changes in IL-1β levels after toxin administration [18]. Furthermore, the proinflammatory cytokines TNFα and IL-1β are involved in DAergic neuronal death in MPTP-treated mice [19]. Together, these data indicate a close association between MPP^+-induced microglial activation and the degeneration of DAergic neurons.

Recent investigations have disclosed the powerful properties of various natural polyphenols against oxidative stress in several cellular and *in vivo* paradigms of neurodegenerative diseases [20–23]. In particular, quercetin, a flavonoid possessing free radical scavenging properties, may protect against oxidative injury by its ability to modulate intracellular signals and promote cell survival [24]. Several studies suggest its potential as a cardioprotective, anticarcinogenic, antioxidant, and antiapoptotic molecule (see references in [25]). Quercetin also exerts a protective effect against microglia activation and NO production and defends DAergic cells against inflammatory damage induced by the potent inflammatory molecule lipopolysaccharide (LPS) [26, 27].

Sesamin as well as sesamol and sesaminol, the other 2 primary compounds in sesame seeds, is likely responsible for the increased stability of sesame oil against autooxidation and the development of rancidity caused by free radicals [28]. Sesamin is also recognized for its positive physiological outcomes, such as hypocholesterolemic and antihypertensive actions, regulation of lipid and alcohol metabolism in the liver [29–31], and protection against oxidative stress and inflammation in PC12 cells [25, 32]. Currently, no data on the effects of natural antioxidant molecules against MPP^+-induced neuroinflammation have been reported.

The objective of this study was to investigate the influence of quercetin and sesamin on MPP^+-induced inflammation in a microglial-neuronal coculture system. Our results demonstrate that quercetin and sesamin reduce the gene expression and protein concentrations of 3 proinflammatory cytokines (IL-6, IL-1β, and TNFα) in N9 microglial cells. Also, quercetin and sesamin decrease inducible nitric oxide synthase (iNOS) protein expression and $^{\bullet}O_2^-$ production and rescue neuronal PC12 cells from glial-evoked apoptotic death.

2. Materials and Methods

2.1. Drugs and Chemicals. All reagents and chemicals were purchased from Sigma (St. Louis, MO) unless stated otherwise.

2.2. Cell Culture and Treatments. PC12 cells, obtained from the American Type Culture Collection (Rockville, MD), were maintained in a humidified environment at 37°C and 5% CO_2 atmosphere. They were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated horse serum (HS), 5% (v/v) heat-inactivated fetal bovine serum (FBS), and gentamicin (50 μg/mL). Neuronal PC12 cell differentiation was evoked by nerve growth factor-7S (50 ng/mL) in DMEM supplemented with 1% FBS for 5 days, as already described [33]. The microglial cell line N9 (a generous gift from Dr. L. Vallières, Centre de recherche, CHUL, Quebec, QC, Canada) was grown in 10% HS in DMEM nutrient mixture F12-ham (DMEM-F12). To assess the influence of quercetin and sesamin on MPP^+-induced N9 inflammation, the cells were pretreated with quercetin (0.1 μM) or sesamin (1 pM) for 3 h and then exposed to MPP^+ (500 μM) for 12 or 24 h. Quercetin and sesamin concentrations in these experiments were determined by previous dose-response curves and kinetic studies [25, 32, 33]. All experiments were performed in medium with charcoal-stripped serum to remove steroids from the medium.

Neuronal PC12 cells and N9 microglia were cocultured to study the impact of MPP^+-activated microglia on the survival of neuronal PC12 cells. N9 microglial cells were grown in culture inserts (pore size 0.4 μm, BD Falcon, Oakville, ON, Canada); then, MPP^+ was added, and inserts containing N9 cells were transferred on neuronal PC12 cells grown previously on coverslips. In this coculture system, microglial cells communicate with neuronal PC12 cells through the semipermeable membrane, avoiding direct contact between the 2 cell populations [26, 34]. The PC12 supernatant was collected 24 h later for cell death measurement, according to the lactate dehydrogenase (LDH) cytotoxicity test and DNA fragmentation assay described below. To control for possible MPP^+ crossing the insert membrane and causing neuronal PC12 death, we performed control experiments on inserts without microglia and after treatment of the medium with MPP^+.

2.3. Cytotoxicity Measurements. Cytotoxicity was evaluated in control and MPP^+ conditions by colorimetric assay, which is based on the measurement of LDH activity released from damaged cells into the supernatant, as already described [33]. LDH, a stable cytoplasmic enzyme present in all cells, is rapidly released into the cell culture supernatant upon plasma membrane damage. Enzyme activity in the culture supernatant correlates with the proportion of lysed cells [35]. Briefly, 50 μL of cell-free supernatant served to quantify LDH activity by measuring absorbance at a wavelength of 490 nm in a microplate reader (Thermo Lab Systems, Franklin, MA). Total cellular LDH was determined by lysing the cells with 1% Triton X-100 (high control); the assay medium functioned as a low control and was subtracted from all absorbance measurements:

$$\text{Cytotoxicity (\%)} = \frac{(\text{Experimental value} - \text{Low control})}{(\text{High control} - \text{Low control})} \times 100. \quad (1)$$

2.4. DNA Fragmentation Analysis. DNA fragmentation was assessed with the single-stranded DNA (ssDNA) apoptosis

ELISA kit (Chemicon International, Temecula, CA). This procedure is based on selective DNA denaturation by formamide in apoptotic cells but not in necrotic cells or in cells with DNA damage in the absence of apoptosis. The detection of denaturated DNA was performed with a monoclonal antibody to ssDNA. The staining of ssDNA in early apoptosis was undertaken with a mixture of antibody and peroxidase-labelled secondary antibody. The reaction was then stopped, and ssDNA fragmentation was quantified by measuring absorbance at a wavelength of 405 nm in a microplate reader (Thermo Lab Systems). ssDNA was calculated with reference to control conditions. Absorbance of positive and negative controls served as quality control of ELISA.

2.5. Detection of Mitochondrial $^{\bullet}O_2^-$. Microglial cells were grown and treated on collagen-coated circular glass coverslips, and MitoSOX Red (Invitrogen, Burlington, ON, Canada) was deployed to estimate intracellular $^{\bullet}O_2^-$ production. This fluorogenic dye is a highly selective indicator of $^{\bullet}O_2^-$ in the mitochondria of live cells. After treating microglia for 9 h with MPP^+ with or without quercetin or sesamin, the medium was removed and the cells were incubated with MitoSOX Red (5 mM) for 10 min at 37°C (Invitrogen). MitoSOX Red rapidly and selectively targets the mitochondria. Once in the mitochondria, it is oxidized by $^{\bullet}O_2^-$ and exhibits red fluorescence. The cells were washed with Hanks' buffered salt solution and 4′,6′-diamidino-2-phenylindole (DAPI) counterstained all nuclei. Then, the cells were fixed in 4% paraformaldehyde for 6 min at 37°C. Coverslips were mounted with Molecular Probes ProLong Antifade kit (Invitrogen). Images were acquired by Leitz inverted microscope with a high-pressure mercury burner and necessary filters, and analyzed with NIS-Element 2.2 software (Nikon, Mississauga, ON, Canada). To demonstrate MitoSOX Red selectivity, sodium diethyldithiocarbamate (DDC), an inhibitor of superoxide dismutase, served as positive control.

2.6. Electrophoresis and Immunoblot Analysis. N9 cells were grown and treated in 6-well plates. Total proteins were extracted with nuclear extraction kit (Active Motif, Brockville, ON, Canada). Proteins were assessed by bicinchoninic acid protein assay kit (Pierce Biotechnology, Inc., Rockford, IL), and equal amounts were loaded onto 12% sodium dodecyl sulfate-polyacrylamide gel. After electrophoretic separation (180 volts, 45 min), the gels were transferred onto polyvinylidene difluoride membranes (0.22 μm pore size, BioRad, Hercules, CA). The blots were blocked for 1 h at room temperature (RT) in 5% nonfat powder milk. Immunoblotting was performed overnight at RT with anti-iNOS antibody (1 : 50) (StressGen, Biotech, Ann Arbor, MI). The following day, the blots were washed and then incubated with peroxidase-conjugated secondary antibody (1 : 10,000), for 1 h at RT, for development with enhanced chemiluminescence substrate solution.

2.7. Real-Time-Quantitative Polymerase Chain Reaction (RT-qPCR). Total RNA was extracted with Sigma's GenElute Mammalian Total RNA extraction kit. RNA was spectrophotometrically measured for each condition, and 1 μg of total RNA was reverse-transcribed with 25 U of M-MULV reverse transcriptase, 1.5 μM of dNTP, and 10 μM of random hexamers. RT-qPCR was then performed in a MiniOpticon RT-PCR system (BioRad) in 20 μL-sized reactions containing 4 μL of cDNA mixture, 0.3 μM of each forward and reverse primer and 10 μL of iQ SYBR Green Supermix (BioRad). Incubation at 95°C for 3 min was followed by 40 cycles of 15 s at 95°C and 30 s at 61°C. Primers for TNFα (5′-TTCTGTCTACTGAACTTCGGGGTGATCGGTCC-3′ and 5′-GTATGAGATAGCAAATCGGCTGACGGTGTGGG-3′), IL-1β (5′-GCCCATCCTCTGTGACTCAT-3′ and 5′-AGGCCACAGGTATTTTGTCG-3′), IL-6 (5′-TTCCATCCAGTTGCCTTCTT-3′ and 5′-ATTTCCACGATTTCCCAGAG-3′), ubiquitin C (5′-AGCCCAGTGTTACCACCAAG-3′ and 5′-TCACACCCAAGAACAAGCAC-3′), β-microglobulin (5′-ATGGGAAGCCGAACATACTG-3′ and 5′-CAGTCTCAGTGGGGGTGAAT-3′) were designed by BLAST sequences with PRIMER3 web-based software and synthesized at Sigma Genosys (Oakville, ON, Canada). Reactions were performed in duplicate, and 3 independent preparations of cDNA were studied. A 10-fold dilution series was obtained from a random pool of cDNA ranging from $\times$10 to $\times$100,000 dilution. Mean cycle threshold values (Ct) for each dilution were plotted against log10 of cDNA input to generate efficiency plots. The reaction efficiency of each gene assay was calculated according to the equation $E = 10(-1/\text{slope})$, where E was reaction efficiency and "slope" was the slope of the line generated in efficiency plots. All PCR efficiencies were above 90%. In all PCR experiments, post-PCR DNA-melting curve analysis was undertaken to assess amplification specificity. DNA melting was carried out at a temperature ramping rate of 1°C per step with 1 s rest at each step. Relative gene transcription was calculated by the comparative Ct method, using the real-time efficiency values of each gene. cDNA levels among the samples were normalized by the expression of 2 internal control genes: ubiquitin and β-microglobulin. These housekeeping genes were chosen with the geNorm algorithm [36]. A normalization factor was calculated with the geometric mean of the 2 reference genes. The normalized expression of each gene of interest was calculated by dividing the raw quantities of each sample by the appropriate normalization factor [36].

2.8. ELISAs for IL-6, IL-1β, and TNFα. IL-6, IL-1β, and TNFα were measured by specific ELISAs (BioLegend, San Diego, CA). After incubation with MPP^+, with or without quercetin or sesamin, for 24 h, the supernatants were collected for each respective ELISA. Mouse-specific monoclonal antibody (IL-6, IL-1β, and TNFα) was first coated on 96-well plates. Standards and samples were then added to the wells for 2 h, where IL-6, IL-1β, or TNFα were bound to the immobilized capture antibody. A biotinylated anti-mouse detection antibody was added for 1 h, producing an antibody-antigen-antibody "sandwich" to which an avidin-horseradish peroxidase solution was added for 30 min. Finally, a tetramethylbenzidine solution was included for 15 min in the dark. Reaction with horseradish peroxidase

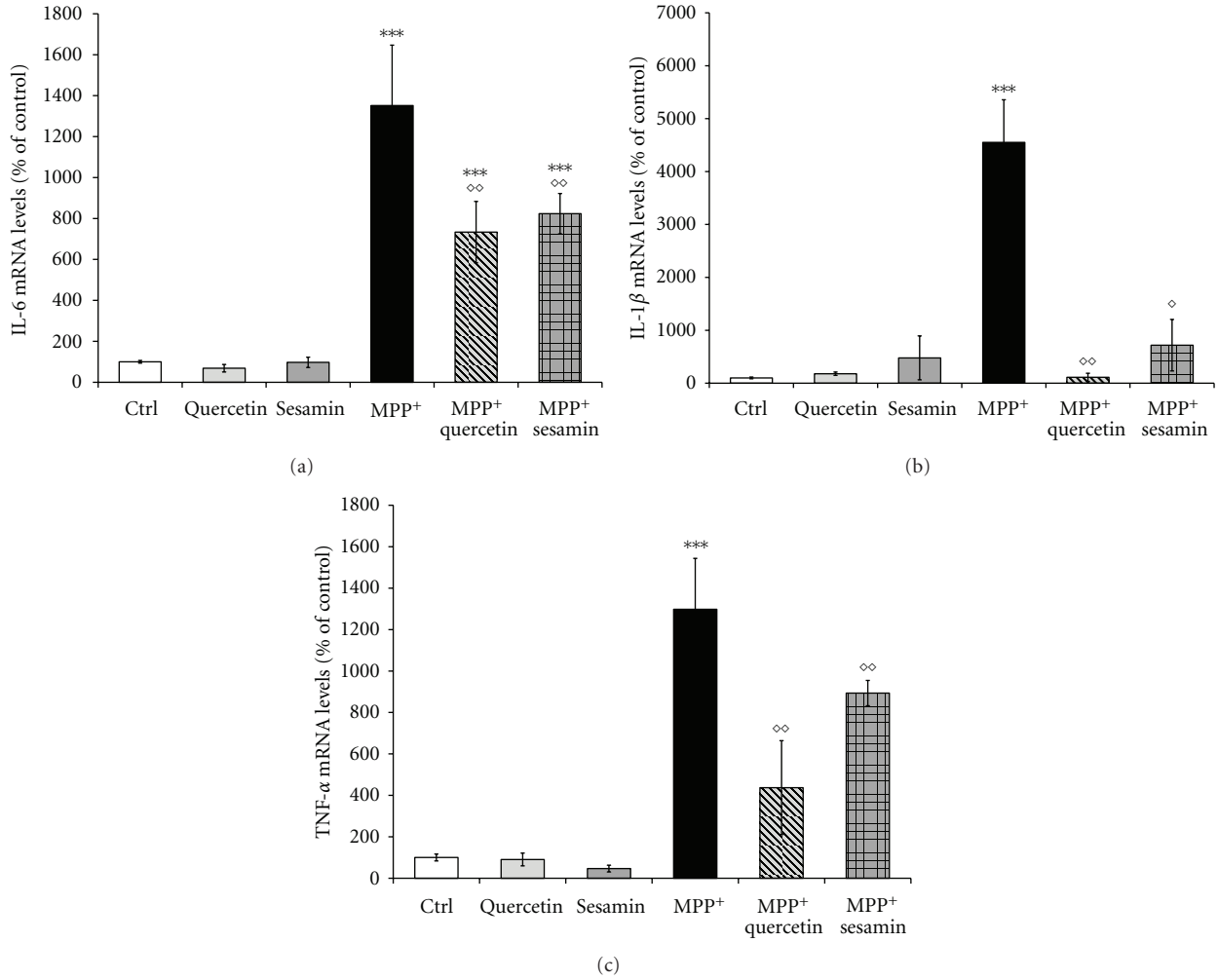

Figure 1: IL-6 (a), IL-1β (b), and TNFα (c) mRNA levels in N9 microglial cells treated first for 3 h with quercetin or sesamin and then with or without MPP$^+$. After 24 h, total RNA was extracted from microglial cells and qRT-PCR was performed. MPP$^+$ clearly induced significant increases of IL-6 (a), IL-1β (b), and TNFα (c) mRNA levels. When N9 microglial cells were treated with quercetin or sesamin 3 h prior to MPP$^+$, we detected significant decreases of IL-6, IL-1β, and TNFα mRNA levels (MPP$^+$ quercetin, MPP$^+$ sesamin). Bars represent the average intensity of the bands ± SEM of 3 independent experiments expressed as % of the controls. $^{***}P < 0.001$ compared to the control (Ctrl), $^{\diamond\diamond}P < 0.01$ and $^{\diamond}P < 0.05$ compared to MPP$^+$, as determined by 1-way ANOVA, followed by Tukey's multiple comparison test.

resulted in conversion of the substrate to a blue-colored product. Addition of 2 N sulfuric acid stop solution yielded a yellow color. Microwell absorbance was read at 450 nm with a microplate reader (Thermo Lab Systems).

2.9. Statistical Analysis. Significant differences between groups were ascertained by 1-way analysis of variance (ANOVA), followed by Tukey's post-hoc analysis with the GraphPad InStat program, version 3.06 for Windows (San Diego, CA; http://www.graphpad.com/). All data, analyzed at the 95% confidence interval, were expressed as means ± SEM from 3 independent experiments. Asterisks indicate statistical differences between the treatment and control condition ($^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$), diamonds denote statistical differences between the treatment and MPP$^+$ condition ($^{\diamond\diamond\diamond}P < 0.001$, $^{\diamond\diamond}P < 0.01$, and $^{\diamond}P < 0.05$), and $^{\circ}$empty circle indicates difference between MPP$^+$-treated cells and their respective control conditions ($^{\circ}P < 0.05$).

3. Results

3.1. Quercetin and Sesamin Decrease MPP$^+$-Induced IL-6, IL-1β, and TNFα mRNA and Protein Concentrations. We measured the expression of the potent proinflammatory cytokines IL-6, IL-1β, and TNFα by RT-qPCR. Figures 1(a), 1(b), and 1(c) show that MPP$^+$ induced N9 microglial cell activation by dramatically increasing these cytokine mRNA levels. No significant difference from the control condition was detected when quercetin and sesamin were administered alone. On the other hand, quercetin or sesamin pretreatment of microglial N9 cells 3 h before MPP$^+$ administration elicited a reduced pattern of IL-6, IL-1β, and TNFα

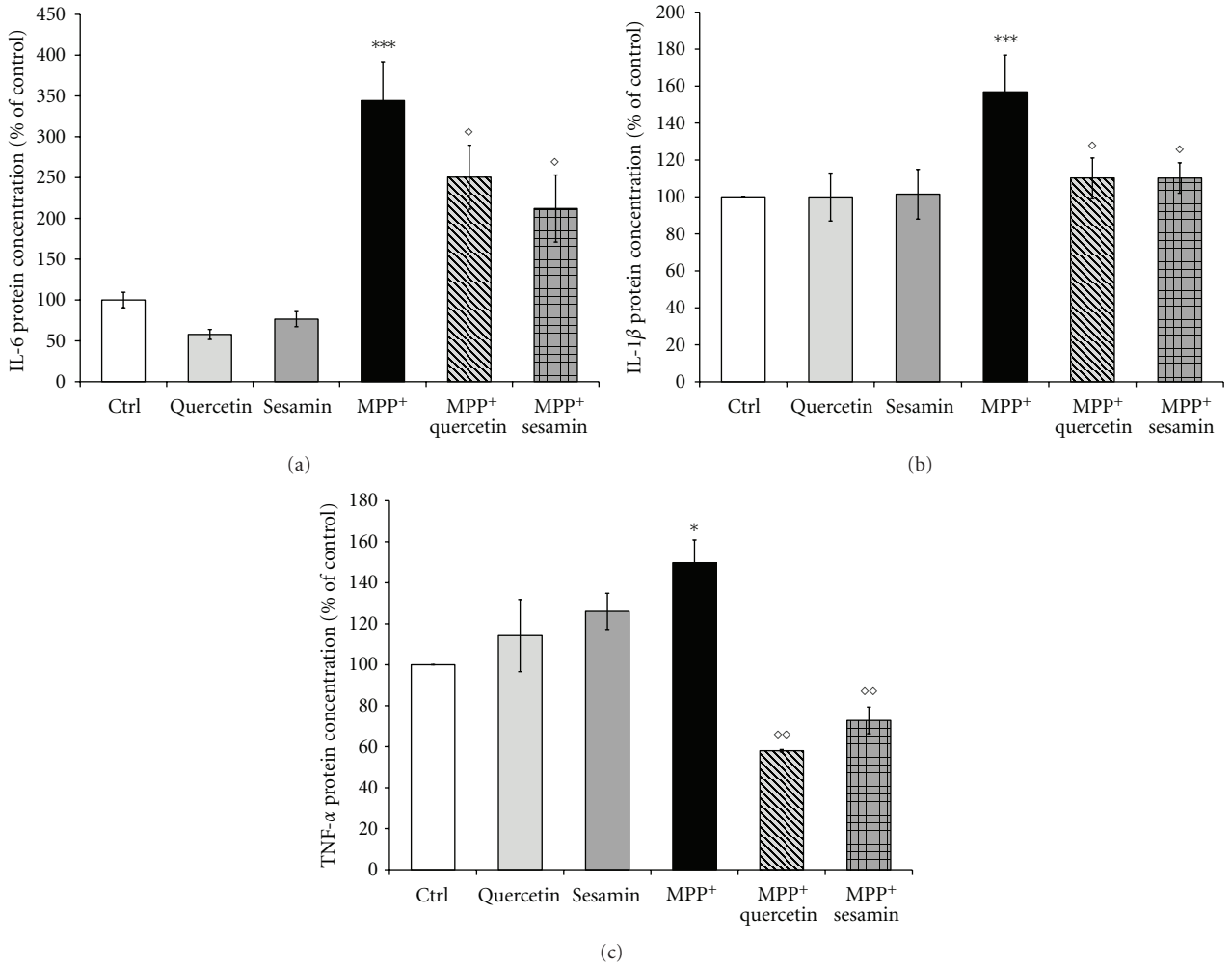

FIGURE 2: IL-6 (a), IL-1β (b) and TNFα (c) protein concentration in N9 microglial cells treated first for 3 h with quercetin or sesamin and then with or without MPP^+. After 24 h, supernatants were collected and ELISA was performed as described in Materials and Methods. MPP^+ induced marked increases of IL-6 (a), IL-1β (b) and TNFα (c) protein concentration. When N9 microglial cells were treated with quercetin or sesamin 3 h prior to MPP^+, we detected significant declines of IL-6, IL-1β and TNFα protein concentration (MPP^+ Quercetin, MPP^+ Sesamin). $N = 3$, $^{***}P < 0.001$ and $^{*}P < 0.05$ compared to the controls (Ctrl), $^{\diamond\diamond}P < 0.01$ and $^{\diamond}P < 0.05$ compared to MPP^+, as determined by 1-way ANOVA, followed by Tukey's multiple comparison test.

gene expression, suggesting that quercetin and sesamin are notably involved in the expression of these cytokines. We also evaluated the protein expression of IL-6, IL-1β, and TNFα by specific ELISAs, as described in Section 2. Our results illustrate that the administration of quercetin or sesamin alone does not modulate IL-6, IL-1β, or TNFα protein expression (Figures 2(a), 2(b), and 2(c)). On the other hand, treatment with MPP^+ alone considerably increases the presence of proinflammatory cytokines, indicating that MPP^+ can induce an inflammatory process in microglial cells. The MPP^+-evoked elevation of IL-6, IL1-β, or TNFα protein expression was strongly attenuated to control levels in microglial cells pretreated with quercetin or sesamin (Figures 2(a), 2(b), and 2(c)), suggesting that these natural substances play a role as anti-inflammatory molecules by impacting both the gene and protein expression of proinflammatory cytokines.

3.2. Quercetin and Sesamin Modulate MPP^+-Induced iNOS Protein Expression. Early studies using iNOS inhibitors provided evidence of their potential as neuroprotective agents in the treatment of PD [37]. Here, we observed a very significant rise of iNOS expression by western blotting in microglia cells after only 1 h of MPP^+ administration (Figure 3(a)). Then, iNOS expression decreased during a 24 h period (Figure 3(a)). We thus analyzed whether quercetin or sesamin might modulate iNOS protein expression after 1 h of MPP^+ administration (Figure 3(b)). Our results illustrate that the polyphenol quercetin and the lignan sesamin consistently decreased MPP^+-induced iNOS expression at 1 h.

3.3. Quercetin and Sesamin Counteract MPP^+-Induced $^{\bullet}O_2^-$ Production in N9 Microglial Cells. To investigate the mechanism underlying the protective properties of quercetin and sesamin against MPP^+ treatment in N9 microglial cells,

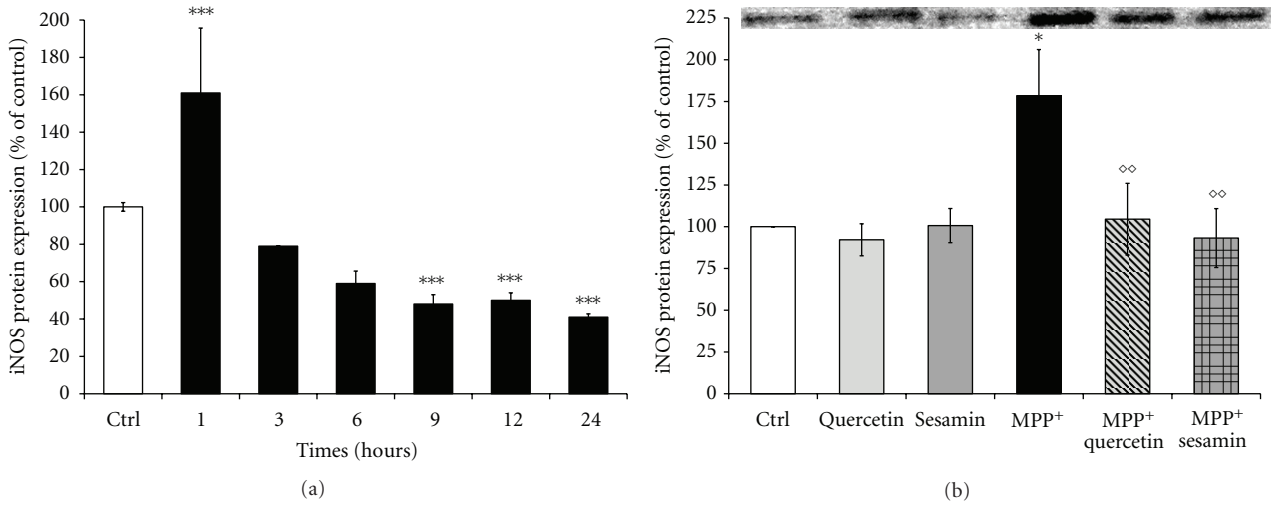

Figure 3: (a) Histogram of kinetic studies showing iNOS protein expression for 24 h in neuronal PC12 cells as revealed by western blotting. Ctrl represents 100% of the controls for each time period. (b) iNOS protein expression after MPP^+ administration with or without sesamin or quercetin. Quercetin or sesamin alone did not alter iNOS protein expression, whereas MPP^+ increased iNOS protein levels by 79% in our cellular paradigm. When sesamin or quercetin was administered prior to MPP^+, a significant decline of iNOS was detected. $^{***}P < 0.001$ and $^{*}P < 0.05$ versus Ctrl; $^{\diamond\diamond}P < 0.01$ versus MPP^+, as determined by 1-way ANOVA, followed by Tukey's multiple comparison test.

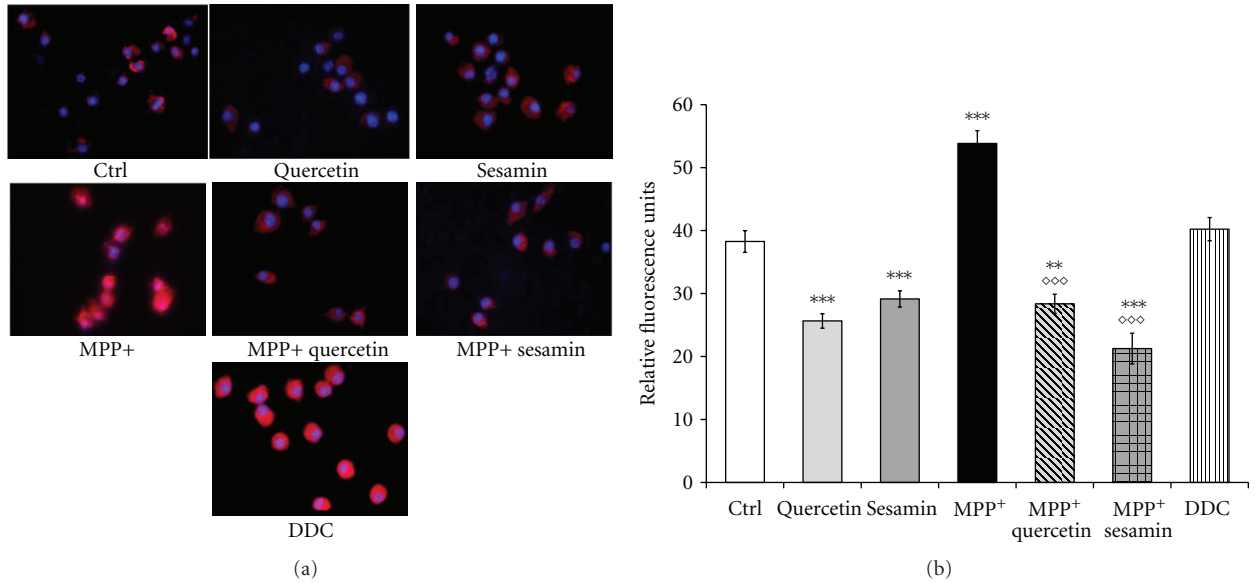

Figure 4: Effects of quercetin and sesamin on MPP^+-induced superoxide anion ($^{\bullet}O_2^-$) in N9 microglia cells. (a) Fluorescence photomicrographs. Ctrl: cells were treated with control medium. quercetin, sesamin: cells were treated with quercetin or sesamin in control medium. MPP^+: cells were treated with 500 μM MPP^+. MPP^+quercetin or MPP^+ sesamin: cells were treated with quercetin or sesamin plus MPP^+. A marked red signal was evident only in neuronal PC12 cells treated with MPP^+. Red fluorescence was less intense in cells treated with control medium (Ctrl) or when quercetin or sesamin was added to MPP^+ medium (MPP^+quercetin or MPP^+sesamin). Magnification 400x. $n = 3$. DDC: sodium diethyldithiocarbamate. (b) Semiquantitative image analysis. $^{***}P < 0.001$ and $^{**}P < 0.01$ compared to the control (CTRL), $^{\diamond\diamond\diamond}P < 0.001$ compared to MPP^+, as determined by 1-way ANOVA, followed by Tukey's multiple comparison test.

we estimated $^{\bullet}O_2^-$ production with MitoSOX Red, a derivative of ethidium bromide, as already described [37], after MPP^+ administration or not, with or without quercetin for 9 h. This time period was considered since ROS, and eventually oxidative stress, are early events in the causative process of cellular death [37]. MitoSOX Red, a fluorogenic dye, was highly selective in detecting $^{\bullet}O_2^-$ in the mitochondria of live cells. Low fluorescence levels were apparent in control microglial cells as well as in cells treated with quercetin or sesamin alone (Figure 4(a): Ctrl, quercetin, sesamin), whereas a marked signal was detected in MPP^+-treated microglial cells (Figure 4(b), MPP^+). Figure 4(b) reports

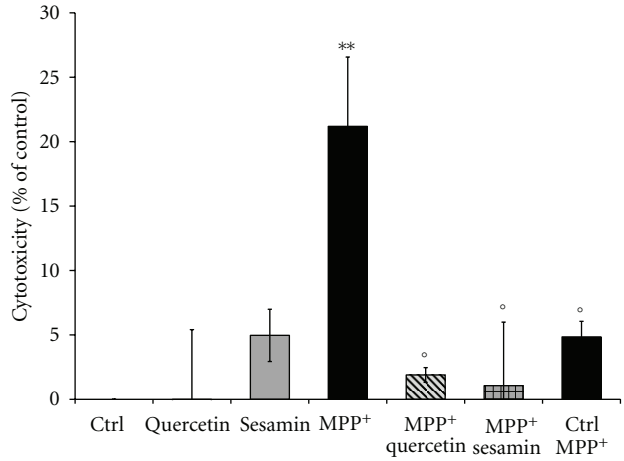

Figure 5: Neuronal PC12 cells were cocultured with MPP^+-activated N9 microglial cells pretreated or not with quercetin or sesamin for 24 h. Then, neuronal PC12 cell supernatants were collected for cell death measurement by the LDH cytotoxicity test. Ctrl: nonactivated microglial cells were placed on neuronal PC12 cells; no neuronal death was detected. MPP^+: MPP^+-activated microglial cells were placed on neuronal PC12 cells; a significant increase of neuronal cell death was apparent, indicating that cytokines produced by microglial cells induce neuronal death. Ctrl MPP^+: MPP^+ was added to the medium of the insert without microglial cells. Pretreatment of N9 cells with quercetin or sesamin prior to MPP^+ clearly reduced neuronal PC12 cell death. Pretreatment of non-MPP^+-activated N9 cells with quercetin or sesamin did not produce cellular death (quercetin, sesamin) $n = 3$. $^{**}P < 0.01$ compared to the control (Ctrl), $^{\circ}P < 0.05$ compared to their respective controls (quercetin, sesamin, or Ctrl), as determined by 1-way ANOVA, followed by Tukey's multiple comparison test.

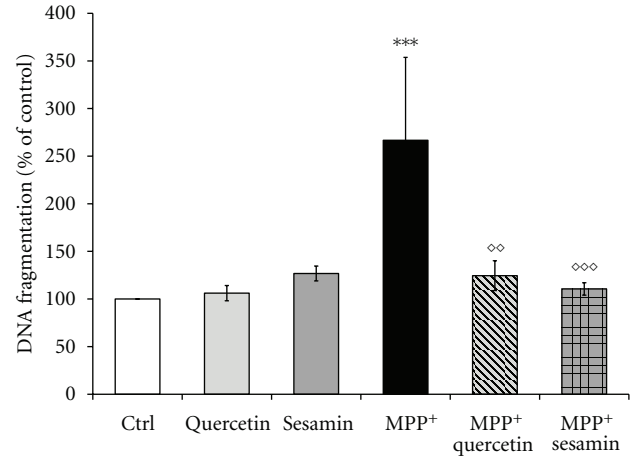

Figure 6: Neuronal PC12 cells were cocultured with MPP^+-activated N9 cells pretreated or not with quercetin or sesamin for 24 h. DNA fragmentation in neuronal cells was detected with a monoclonal antibody to single-stranded DNA (ssDNA). Ctrl: non-activated microglial cells were placed on neuronal PC12 cells; no neuronal death was detected. MPP^+: MPP^+-activated microglial cells were placed on neuronal PC12 cells; a significant increase of neuronal cell death was apparent, indicating that cytokines produced by microglial cells induce neuronal death. Pretreatment of N9 cells with quercetin or sesamin prior to MPP^+ clearly reduced neuronal PC12 cell death. Pretreatment of non-MPP^+-activated N9 cells with quercetin or sesamin did not produce any cellular death. $n = 3$. $^{***}P < 0.01$ compared to the controls (Ctrl), $^{\diamond\diamond}P < 0.01$ compared to quercetin, $^{\diamond\diamond\diamond}P < 0.001$ compared to sesamin, as determined by 1-way ANOVA, followed by Tukey's multiple comparison test.

on the semi-quantitative analysis of mitochondrial $^{\bullet}O_2^-$ depicted in Figure 4(a), revealing high-level fluorescence in the presence of MPP^+ with a very significant reduction ($P < 0.05$ or $P < 0.001$) when microglia cells were treated with quercetin or sesamin.

3.4. Quercetin and Sesamin Reduce MPP^+-Induced Cytotoxicity and Apoptotic Cell Death in Microglial (N9)-Neuronal (PC12) Coculture. To investigate microglial-activated neuronal cell death, we tested a microglial-neuronal coculture system described elsewhere [26]. N9 microglial cells were cocultured in inserts on neuronal, differentiated PC12 cells to evaluate the effect of MPP^+-induced cytokine secretion from microglial cells on neuronal PC12 cell death. The inserts have a pore size of 0.4 μM; thus, they allow cytokines to pass through but prevent cell contact [38]. Neuronal cells placed beneath non-MPP^+-activated N9 cells did not present any significant cell death (Figure 5: Ctrl), whereas neuronal cells cocultured with MPP^+-activated microglial cells displayed a high level of cell death (Figure 5: MPP^+), demonstrating that MPP^+-activated microglia secrete cytokines transported through the membrane insert inducing neuronal death. Figure 5 also reveals that neuronal cell death is diminished to 1.9% when microglial cells are treated with quercetin, and to 1.1% when they were treated with sesamin prior to MPP^+ administration. In addition, our results show no significant neuronal death of PC12 cells exposed to inserts containing MPP^+ without microglial (Ctrl MPP^+), demonstrating that in our cell coculture system, cytokines secreted by microglial cells cross the membrane and elicit neuroinflammation with consequent neuronal PC12 cell death.

To determine whether quercetin and sesamin can protect neuronal DAergic cells from inflammation-induced apoptosis, we also measured ssDNA fragmentation (Figure 6), a marker of late apoptosis. Microglial cells treated with MPP^+ for 24 h manifested a 167% increase in DNA fragmentation—in comparison to control cells (Figure 6)—that was strongly and significantly prevented by quercetin and sesamin (Figure 6). These results disclose that quercetin or sesamin administration to microglial cells can efficiently reduce the apoptotic death of neuronal PC12 cells induced by microglial activation, thus supporting an anti-inflammatory role of these 2 natural molecules.

4. Discussion

Currently, several studies have described microglia activation in the SN *pars compacta* of PD patients and PD animal models [4–6]. As such, neuroinflammation is considered a feature of PD progression and pathogenesis.

Our present data highlight the neuroprotective properties of quercetin and sesamin, 2 natural molecules that reduce the expression of IL-6, IL-1β, and TNFα, 3 cytokines associated with neuroinflammation. We also show that quercetin and sesamin prevent the production of 2 cellular markers of inflammation, iNOS and $^{\bullet}O_2^-$ as well as the apoptosis of DA-producing neurons provoked by microglia stimulation. Our previous studies have already revealed that quercetin and sesamin have neuroprotective, antiapoptotic and antioxidative properties, reducing MPP^+- and LPS-induced neuronal death [25, 26, 32]. Here, we further demonstrate that quercetin and sesamin can also act as potent anti-inflammatory compounds, restraining microglia activation and oxidative stress.

Activated microglial cells contribute to DAergic cell death by releasing cytotoxic inflammatory compounds, such as the proinflammatory cytokines IL-6, IL-1β, TNFα, and interferon-gamma. Among them, IL-6, IL-1β and TNFα have attracted much attention with regard to neuroinflammatory processes in PD [4]. DAergic degeneration induced by MPTP or MPP^+ is linked with an inflammatory response *in vitro* [39] as well as in mouse and primate models of PD [18, 40]. On the other hand, inhibition of microglia activation is neuroprotective [41, 42] and coupled with the attenuation of TNFα expression [43]. The mechanisms by which microglia are activated are not fully understood. However, very recent data on a mouse MPTP model of PD as well as mesencephalic culture support a role of brain angiotensin II as one of the most potent inducers of inflammation and ROS [44, 45].

In this study, we clearly demonstrated that the flavonoid quercetin and the lignan sesamin strongly reduce the expression of proinflammatory cytokines in N9 microglia cells, indicating an interesting anti-inflammatory role of these natural molecules. We also analyzed the effects of quercetin and sesamin on parameters of oxidative cell distress. Activated microglia and excessive NO production by the high-output NO-synthesizing enzyme iNOS are observed in various neurological diseases, including PD [46]. NO is an apoptosis inducer, and iNOS is a key enzyme that produces large quantities of NO. Quercetin exerts a broad inhibitory effect on iNOS gene expression [27]. Sesamin and sesamin metabolites are known to induce endothelial NOS (eNOS) and thus have a significant antihypertensive function [30]. In this study, we demonstrated that quercetin and sesamin markedly reduced MPP^+-evoked upregulation of iNOS expression in microglial cells, corroborating previous data from our group and others [32, 47].

It should be noted that, in our microglial cellular system, MPP^+ increased iNOS production, after only 1 h of administration, and iNOS protein expression then declined constantly. This might be explained by early iNOS production followed by other intracellular apoptotic mechanisms. Certainly, in-depth studies should be performed to analyze the kinetics of iNOS production in this particular cellular system. Several investigations have determined that quercetin and sesamin can decrease ROS production to near-normal levels in various cellular systems [21, 48]. With MitoSOX Red, a selective indicator of mitochondrial $^{\bullet}O_2^-$ production, we illustrated an increase of fluorescence, when MPP^+ was administered alone, and a substantial reduction with quercetin or sesamin treatment, supporting a potent scavenging role of quercetin and sesamin.

Microglia activation leads to increased production of cytokines that could mediate neuronal apoptosis and precede DAergic nigrostriatal neuron degeneration in a PD mouse model [49]. In this study, we show that quercetin and sesamin reduce the cellular death induced by MPP^+ administration. We also document that quercetin and sesamin are potent modulators of apoptosis, opposing MPP^+-induced DNA fragmentation. Our results demonstrate that when neuronal PC12 cells are cocultured with MPP^+-treated N9 cells, their levels of cellular death increase to 267%. Quercetin or sesamin administration to MPP^+-activated microglia reduces apoptotic DAergic neuronal PC12 cell death to 125% and 112%, respectively.

It should be noted that, although natural polyphenols are being studied intensively *in vitro* and *in vivo* for their neuroprotective properties, our knowledge about their bioavailability and possible target organs is far from being complete ([50] for review). In mammals, flavonoids, such as quercetin, as well as the lignan sesamin, are absorbed in the gut and that their bioavailability is much greater than previously believed [51–53]. Flavonoid concentrations in human plasma vary from 3 to 30 microM and certainly more, so if red blood cell-associated flavonoids are taken into consideration [54]. In addition, polyphenols can cross the blood-brain barrier to varying degrees depending on their chemical structure [55–57]. Recent studies have reported that their bioavailability in the nervous system may be improved by designing specific synergies between orally consumed polyphenols ([58] for review). Recent critical and comprehensive reviews report that quercetin and its metabolite isorhamnetin are found in the brain of rats and pigs in measurable levels ([50, 59] and references within), while nowadays less is known regarding the degree of brain bioavailability of sesame lignans. Sesamin can be converted to the mammalian lignans (enterodiol, enterolactone, and sesamol) by the intestinal microflora and is found in circulating blood. Recent results report that in rats orally administered sesamin can improve oxidative stress induced by kainic acid-induced status epilepticus and middle cerebral artery occlusion [60, 61]. In this study, we have used quercetin at 10^{-7} M which is a relative high concentration compared to that reported *in vivo* in the pmol and nmol/g/tissue range [50]. However, for sesamin, we used 10^{-12} M concentration that should be physiologically attainable *in vivo*.

Currently, more and more *in vivo* studies point out the importance of the biotransformation of natural molecules and the magnitude of bioavailability of the parental molecule and/or its metabolites for the prevention of human diseases [59]. With respect to these *in vivo* studies, our *in vitro* experiments document for an anti-inflammatory response with both quercetin and sesamin in a glial-neuron coculture system. Although far from an *in vivo* trial, it remains one of the best *in vitro* paradigms to study the possible relationship between microglial production of proinflammatory cytokines and neuronal cellular death.

Nevertheless, the information obtained with this study is also valuable providing new insights into the cellular mechanisms of natural compounds as preventive and/or complementary therapies for human diseases.

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